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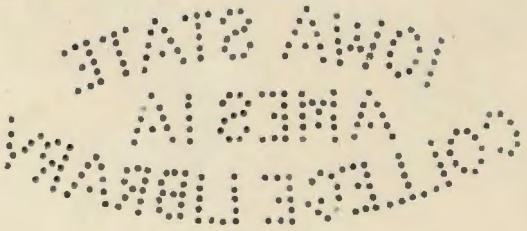

THE EFFECT OF PANTOTHENATE DEFICIENCY ON *TRYPANOSOMA LEWISI* INFECTION IN THE RAT

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It has been reported by Becker, Manresa, and Johnson (1943) that the multiplicative period of *Trypanosoma lewisi* in the rat's blood was inordinately lengthened by feeding the host a ration deficient in pantothenic acid, and that as a result there was an exaltation of parasite density, sometimes resulting in death of the host. Because Bartonella-like bodies were frequently encountered in stained blood films from the affected rats, and blood picture and spleen weight were suggestive of bartonelliasis, there was no assurance that the altered course of the infection was not due primarily to intercurrent infection rather than to the deficiency itself. In a reinvestigation of the effect of pantothenic acid deficiency on the course of *T. lewisi* infection it has been possible to eliminate the factor of intercurrent infection. In the new work the deficiency was effected by excluding the vitamin from the construction of the ration instead of by dry-heating as before. Observations were also made on the effect of the parasitemia on weight gains and on red and white cell counts.

MATERIAL AND METHODS

Cultures of the strain of *Trypanosoma lewisi* employed were obtained from the Army Medical School, Washington, D. C. The strain was supposed to be the one obtained from the senior author in 1943, then carried on in rats at that place. The strain at first exhibited greater multiplicative powers than hitherto, for counts per cubic millimeter of blood often exceeded those obtained before 1943. Bartonella did not appear in any of the pantothenate-deficient rats inoculated with trypanosomes in the blood of the rats seeded from the cultures, nor in any of the rats through which the trypanosomes were subsequently passed. The loss of this microorganism was possibly due to its inability to grow in the blood agar culture medium.

The host employed was the Wistar A rat. The colony is louse-free. One litter was used in each experiment. The rats were started on the test rations when the mean weights of the litters averaged from 59 to 79 grams (see parentheses under experiment numbers in Table 1). Rats of these weights did not show the extreme symptoms of pantothenic acid

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TABLE 1

WEIGHT INCREASES AND TRYPANOSOME NUMBERS PER CUBIC MILLIMETERS OF BLOOD FOR RECIPIENTS AND NONRECIPIENTS OF PANTOTHENATE (P.A.)

Experiment Number	Rat No.	Sex	P.A.	Weight Increases (grams)*						Trypanosome Numbers (10,000)†				
				P	7D	10D	12D	15D	17D	7D	10D	12D	15D	16D
1	1	M	−	36	....	68	69	....	....	....	11	7	....	....
(73 grams)	2	M	−	50	....	72	69	....	....	....	187	435	....	....
	3	F	−	30	....	55	51	....	....	....	2	2	....	....
	4	F	−	39	....	62	64	....	....	....	76	70	....	....
	5	M	+	44	....	109	119	....	....	....	53	67	....	....
	6	F	+	42	....	86	94	....	....	....	14	10	....	....
	7	F	+	39	....	69	77	....	....	....	3	0	....	....
	8	M	+	54	....	120	126	....	....	....	0	0	....	....
2	9	F	−	39	44	42	45	....	....	....	31	21	....	....
(59 grams)	10	M	−	45	58	58	57	....	....	....	174	141	....	....
	11	F	+	51	75	80	94	....	....	....	32	24	....	....
	12	M	+	58	103	120	134	....	....	....	2	1	....	....
	13	F	+	51	77	83	94	....	....	....	22	20	....	....
3	14	M	−	16	27	23	29	29	....	....	106	89	126	....
(62.5 grams)	15	M	−	26	32	38	35	26	....	....	111	84	118	....
	16	F	−	30	38	42	45	47	....	....	81	79	158	....
	17	F	+	57	91	97	93	107	....	....	6	2	1	....
	18	F	+	53	75	86	97	104	....	....	34	25	11	....
	19	M	+	65	109	127	143	157	....	....	5	5	4	....
4	20	M	−	39	54	61	65	67	....	....	80	95	52	....
(70.5 grams)	21	M	−	36	57	66	73	74	....	....	66	64	30	....
	22	F	−	37	61	64	73	70	....	....	23	21	19	....
	23	F	−	39	52	58	67	65	....	....	19	19	15	....
	24	M	+	62	97	96	....	....	....	....	9	12	4	....
	25	M	+	68	108	103	....	....	....	....	0	0	0	....
	26	F	+	52	83	81	....	....	....	....	0	0	0	....
	27	F	+	54	85	87	....	....	....	....	15	15	8	....
5	28	F	−	40	42	54	52	....	50	2	17	13	....	4
(67.4 grams)	29	M	−	41	44	48	51	....	50	10	48	27	....	17
	30	M	−	47	46	52	53	....	56	4	41	25	....	13
	31	F	−	38	40	49	50	....	50	8	19	20	....	8
	32	F	+	42	56	63	70	....	79	3	6	3	....	3
	33	F	+	44	62	70	80	....	89	3	4	1	....	1
	34	M	+	40	53	60	67	....	75	4	4	1	....	1
	A	F	−	37	55	66	76	....	82	Not inoculated				....
	B	M	−	41	48	58	61	....	63	Not inoculated				....
	C	F	−	37	42	53	56	....	58	Not inoculated				....
	D	M	−	45	57	68	73	....	75	Not inoculated				....
6	35	M	−	53	67	76	....	....	....	7	13	....	6	....
(79 grams)	36	F	−	41	52	62	....	....	....	13	17	....	1	....
	37	F	−	30	39	38	....	....	....	13	30	....	18	....
	38	M	−	49	57	65	....	....	....	15	5	....	3	....
	39	F	+	43	57	80	....	....	....	1	+	....	0	....
	40	F	+	47	61	67	....	....	....	+	+	....	0	....
	41	F	+	43	62	71	....	....	....	8	9	....	6	....
	42	M	+	57	79	89	....	....	....	+	+	....	+	....

* P = Preparation Period (12–14 days)
7D = Seventh Day of Infection
10D = Tenth Day of Infection
12D = Twelfth Day of Infection, etc.
† Less than 5,000 indicated by +.

deficiency obtainable in younger rats, but nevertheless the course of the *T. lewisi* infection was affected. The experimental rats were inoculated intraperitoneally with about 100,000 trypanosomes each.

The deficient diet had the following composition: sucrose, 640 g.; vitamin-free casein, 220 g.; hydrogenated vegetable oil, 80 g.; cod liver oil, 20 g.; salt mixture, 40 g. The following amounts of crystalline vitamins were added to each 1,000 g. of the above: thiamine hydrochloride, 8 mg.; pyridoxine hydrochloride, 10 mg.; riboflavin, 10 mg.; inositol, 100 mg.; choline chloride, 1 g.; para amino benzoic acid, 10 mg.; nicotinic acid, 20 mg. The reference diet was the same supplemented daily with 200 μg. calcium pantothenate per rat.

Trypanosome counts were made some by the method of Taliaferro (1924) and some by that of Kolmer (1915). Red- and white-cell counts were made by standard haemocytometer methods. Differential white cell counts were made on blood films stained with Wright's. The presence or absence of division forms of *T. lewisi* was determined by inspection of the stained blood films. Uniform size and form are, of course, indicative of adulthood, or the non-multiplicative condition, while variability of size, form, and stainability, as well as concrete evidence of division of cell organelles, indicate reproduction.

EXPERIMENTAL DATA

Data composed of weight increases of the hosts and numbers of trypanosomes per cubic millimeter of blood are summarized in Table 1. It is apparent in all six of the experiments, in which the rats of six litters were employed, that the deficient rats failed to make the weight gains registered for the recipients of pantothenate. On the other hand, the trypanosome population in the deficient rats attained heights not equaled in the normal rats. It is true, however, that the trypanosome numbers in individuals of the latter often exceeded certain ones of the former, and that there were individuals which did not seem to exhibit decreased resistance by reason of the decreased dietary deficiency. An example of the latter is Rat No. 3, harboring a population of 2,000 trypanosomes per cubic millimeter of blood on the tenth and twelfth days. It is entirely possible, however, that such a rat, if it had been fed pantothenate, would have reacted either like Rat No. 8, which did not exhibit trypanosomes in the circulating blood, or like Rats Nos. 40 and 42 in which they were seen but not in sufficient numbers to be counted.

An analysis of the trypanosome numbers appearing in Table 1 shows the following means of the maximum populations (in tens of thousands) attained in the nonrecipients of pantothenic acid (A) and the recipients (B):

Experiment 1, (A) 131 and (B) 21; Exp. 2, (A) 102 and (B) 19; Exp. 3, (A) 99 and (B) 15; Exp. 4, (A) 51 and (B) 7; Exp. 5, (A) 32 and (B) 5; Exp. 6, (A) 19 and (B) 2.

Thus the means for the nonrecipients were from 5.4 to 9 times as high as those for the recipients. The record shows a strange decrease in both

sets of means with each succeeding experiment. The true explanation of this phenomenon is not yet known to the writers, but it may be that as the microorganism was passed from rat to rat of our strain it regained that tendency, which was commented upon in the previous report, to appear only in small numbers in the circulating blood.

Microscopic examinations of stained blood smears confirmed that, as in the previous work, the multiplicative phase of the *T. lewisi* infection was abnormally prolonged in the deficient rats. Without exception, the parasite population attained adulthood (defined above) by the eighth, ninth, or tenth day in the pantothenate recipients, whereas there were a number of exceptions to this behavior among the deficients, as indicated by flagellates with dividing or divided parabasal bodies and nuclei, double flagella, and variability in body size and shape. The most marked exceptions occurred in Rats 2, 4, 10, 14, 15, and 16, in whose blood evidence of division forms was detected at least as late as the fourteenth or fifteenth days. Rats 14 and 16 presented especially interesting cases, for division forms appeared in them continuously from May 10 (fourth day of the infection) to July 11 (sixty-sixth day). The condition of the rats became so severe by the latter date that, in order to save them, the stock-growing ration was fed. The condition of the rats rapidly improved, as indicated by increasing weight, hair growth, and better care of the coat, but size variability persisted in the flagellate population for two weeks.

Rats 14 and 16 were of special interest for another reason: their parasitemias apparently reached a peak on about the fifteenth day and steadily declined thereafter, although division forms persisted. The day of the infection (parenthesis) and trypanosome numbers in hundreds of thousands per cubic millimeter of blood for rats 14 and 16, respectively, were recorded as follows: (10) 1,060 and 810; (12) 890 and 790; (15) 1,260 and 1,580; (18) 505 and 239; (22) 375 and 191; (24) 351 and 261; (34) 72 and 249; (40) 140 and 130; (66) 50 and 42.

Only two deaths due to trypanosomiasis occurred among the deficients, Rat 2 on the fifteenth day of its infection, and Rat 15 on the seventeenth. Division forms were found in the blood of both rats on the day preceding death, and both showed evidences of extreme red cell anemia; namely, pallor of eyes and mucous membranes, and occurrence of normoblasts and reticulocytes in the smears. Counts were not made on Rat 2, but in Rat 15 the red cell count dropped from 6 million on the day of inoculation to 1.2 million on the fifteenth day. It was a curious coincidence that on the latter date the numbers of trypanosomes and red cells per cubic millimeter of blood were practically identical.

It will be noted in Table 1 that the weight increases were in general considerably higher in the infected pantothenate recipients than in the infected deficients. Experiment 5 shows that this is by no means entirely due to the vitamin deficiency, for the weight gains up to the seventeenth day were considerably higher in the uninfected deficients than in the infected deficients.

The development of red cell anemia in the pantothenate series was

found to be a common occurrence. A litter of eleven young rats averaging about 67 grams was selected for special study. Nos. 28–31 and A–D were started on the pantothenate deficient ration, and Nos. 32–34 on the same plus pantothenate. All except A–D were inoculated with *T. lewisi* on the fourteenth day. Red cell counts on the inoculation date and the seventh, tenth, thirteenth, and eighteenth day of the ensuing infection are recorded in Table 2. The trypanosome counts on the seventh, tenth, twelfth, and sixteenth days are recorded in Table 1.

Trypanosome numbers were, as expected, considerably higher in the deficient series than in the normals (Table 1). Likewise, as previous observations had led us to expect, red cell anemia developed in all four infected deficients, but not to any considerable degree in the others. In certain other instances we had found anemia commencing as early as the seventh day, and in this series likewise it seemed to commence about that date and to become extreme by the tenth and thirteenth days. The severity of the anemia in these cases was in direct proportion to the parasitemia, an impression we had gained from previous observations. Anemia did not develop in the four uninfected deficient rats.

The differential white cell counts for the deficient series (Rats 28–31) showed an increase in neutrophils at the expense of the lymphocytes on the seventh, tenth, and thirteenth days, with some readjustment toward the normal by the eighteenth day (Table 2). There was a higher eosinophilia in the normal series than in the deficient or uninfected series, but the observations are too few to warrant definite conclusions. The evidence for a basophilia in the deficient series is, however, strong (*see* Table 2). The increase in total white cell counts was more marked and prompt in the normal than in the deficient series.

SUMMARY AND CONCLUSIONS

Since our previous work on the effect of pantothenic acid deficiency on *Trypanosoma lewisi* infection was complicated by intercurrent Bartonella infection, another attack on the problem was made with a Bartonella-free line of the microorganism and louse-free rats. In the new work the deficiency was produced by withholding pantothenic acid from the diet rather than by the drastic process of dry-heating previously employed. Also, a complete vitamin supplement was fed, save for the absence of pantothenate in the deficient series.

In each of the six test experiments, employing in all twenty-one young rats in the deficient series and the same number in the normal series, the parasitemia was abnormally exalted in the hosts from whom pantothenate was withheld. Likewise, the multiplicative period of the infection was abnormally prolonged in the deficient hosts whose parasite populations were the most enhanced.

Other aspects of the infection in pantothenate deficient rats were (1) anemia, (2) neutrophilia, (3) basophilia, (4) less than normal and more delayed increase in total white cell count, (5) either reduced growth

TABLE 2

BLOOD CELL CHANGES IN INFECTED NONRECIPIENTS OF PANTOTHENATE (28–31), INFECTED RECIPIENTS (32–34), AND UNINFECTED NONRECIPIENTS (A–D)

(Started on diets June 14 when 40 days of age and infected June 28)

Rat No.	P.A.	Date	Red Cells (1,000,000)	White Cells (1,000)	Lymph. %	Neutr. %	Mono. %	Eos. %	Bas. %
		6–28	7.2	6.6	72	20	7	1	.....
		7–5	6.8	4.2	60	33	6	.....	1
28...	–	7–8	4.2	6.3	59	36	3	1	1
		7–11	4.4	6.8	62	34	2	.....	2
		7–16	5.4	6.8	61	31	6	.....	2
		6–28	7.2	4.9	83	12	5	.....	.....
		7–5	6.1	4.9	56	40	2	1	1
29...	–	7–8	2.4	4.5	54	40	1	1	4
		7–11	1.8	10.7	53	45	.....	.....	2
		7–16	4.3	6.2	60	37	3	.....	.....
		6–28	9.9	4.7	78	20	2	.....	.....
		7–5	7.7	4.4	62	34	2	.....	2
30...	–	7–8	3.5	5.6	60	40	.....	.....	.....
		7–11	2.5	10.4	61	36	1	.....	2
		7–16	3.8	9.0	74	21	4	1	.....
		6–28	6.2	5.1	74	22	4	.....	.....
		7–5	5.8	4.7	44	51	4	.....	1
31...	–	7–8	4.5	4.3	75	23	2	.....	.....
		7–11	3.0	6.9	38	56	3	.....	3
		7–16	2.8	7.8	72	24	4	.....	.....
		6–28	6.9	5.0	56	42	1	1	.....
		7–5	7.5	8.9	50	41	8	1	.....
32...	+	7–8	6.7	11.0	86	12	2	.....	.....
		7–11	5.9	9.0	68	25	5	2	.....
		7–16	5.8	7.6	86	12	2	.....	.....
		6–28	4.9	7.0	74	22	4	.....	.....
		7–5	6.2	8.9	78	20	2	.....	.....
33...	+	7–8	6.1	13.3	88	11	1	.....	.....
		7–11	4.6	9.8	64	28	6	2	.....
		7–16	7.0	13.0	73	21	6	.....	.....
		6–28	5.6	6.5	69	19	11	1	.....
		7–5	6.9	7.0	74	24	1	1	.....
34...	+	7–8	7.0	10.5	75	23	1	1	.....
		7–11	6.9	9.0	66	30	4	.....	.....
		7–16	7.0	8.8	70	26	2	1	1
		6–28	7.9	6.4	89	9	2	.....	.....
		7–5	7.4	8.4	84	10	6	.....	.....
A....	–	7–8	6.2	6.9	85	10	5	.....	.....
		7–11	7.2	5.9	81	14	5	.....	.....
		7–16	7.5	6.0	72	21	5	1	1
		6–28	6.0	4.8	69	23	8	.....	.....
		7–5	7.2	6.9	68	24	7	.....	1
B....	–	7–8	7.4	8.3	78	18	4	.....	.....
		7–11	8.8	7.7	68	26	6	.....	.....
		7–16	8.7	7.8	71	22	7	.....	.....

TABLE 2—*Continued*

Rat No.	P.A.	Date	Red Cells (1,000,000)	White Cells (1,000)	Lymph. %	Neutr. %	Mono. %	Eos. %	Bas. %
		6–28	7.6	3.6	76	20	3	1	.....
		7–5	7.5	9.6	79	20	1	.....	.....
C....	–	7–8	7.4	8.1	81	15	4	.....	.....
		7–11	7.6	6.0	69	25	5	1	.....
		7–16	8.1	6.5	68	27	5	.....	.....
		6–28	7.3	5.3	85	12	3	.....	.....
		7–5	6.8	7.0	76	17	6	1	.....
D....	–	7–8	6.1	7.2	59	34	7	.....	.....
		7–11	7.5	7.4	60	36	4	.....	.....
		7–16	7.8	7.6	68	28	3	.....	1

rate of host or loss of weight and, (6) in extreme cases, death of the host.

Since anemia, changes in total and differential white cell counts, and sudden adverse effects on growth did not occur in the uninfected deficient controls, it can be said that *T. lewisi* may become a pathogen in pantothenate deficient rats.

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CURVE FITTING: AN ART OR A SCIENCE?

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Graphical curve fitting is done in accord with the judgment of the fitter: the curve may reflect his judgment more faithfully than it does the fitted points. Often the data are not sufficiently plentiful to afford any objective comparison between curves fitted by eye and by least squares; hence, the recent report of a successful graphical fitting with 216 sets of observations (Becker, Carter, Burks, and Kaleita: this *Journal,* Volume 20, No. 4, July, 1946, pages 403–13) was seized upon as an opportunity to compare methods.

After abandoning a direct fitting of the data to the desired dependent variable, plasma atabrine, A.P., the authors (Becker, *et al.*) discovered a quantity, $F = A.U./(T.A. \cdot A.P.)$, which followed a notable pattern in the plane of the two independent variables, urinary atabrine, A.U., and titratable acidity of urine, T.A. So, they marked off the plane into a series of bands within each of which F was approximately constant. By means of this chart, the two field measurements, A.U. and T.A., determined a value of F_m from which the plasma atabrine was estimated as,

$$C.A.P. = \frac{A.U.}{(T.A.)\ (F_m)}$$

In this article two features will be discussed, the fitting of F and the original problem of estimating A.P.

THE FITTING OF F

The authors ran into two difficulties in fitting F: one group of six points lay so close to the T.A.-axis that there was some skepticism as to the validity of the results, and one point was found at the opposite extreme, very close to the axis of A.U. These seven points were, in effect, omitted from the graph. It was partly to learn the sources of these difficulties that the surface now to be described was fitted to the 216 points.

As a first trial, it was deemed adequate to fit the general quadratic function, the result being,

$$F = 108 + 0.028\ A.U. - 53T.A. - 0.0000004\,(A.U.)^2 + 5.4\,(T.A.)^2 - 0.003\,(A.U.)\,(T.A.)$$

Multiple R^2 was only 0.3581. One of the authors' difficulties was still in evidence: the 216th point, lying close to the vertical axis A.U., had the deviation from the fitted surface, $888 - 176 = 712$. This single point was responsible for 71 per cent of the entire sum of squares of deviations

from regression. For the objectives in view it seemed wise to omit this point as the authors effectively did. Upon doing this the equation became

$$F = 85 + 0.023\ \text{A.U.} - 37\text{T.A.} - 0.0000003\,(\text{A.U.})^2 + 3.61\,(\text{T.A.})^2 - 0.002\,(\text{A.U.})\,(\text{T.A.}),\ \text{with}\ R^2 = 0.5941.$$

Some light is now thrown on the other difficulty that the authors mentioned. The calculated values of F for the first six points are,

$$-8, -6, 1, -6, -3, \text{and } 10$$

The four negative F's lead to negative estimates for A.P. From this, together with many other negative F's and large deviations from regression, it was inferred that there is no polynomial function of A.U. and T.A. (with a moderate number of terms) which is suitable for the data in hand; this may account for the hesitancy of the authors to use the points 1–6.

From the successful estimation of A.P. reported in the second part of this article, the present writers guessed that the logarithms of the variables might be more appropriate for fitting the data. The linear regression in logarithms,

$$\log F = 1.421 - 0.8334\,(\log \text{T.A.} + 1) + 0.6117\,(\log \text{A.U.} - 1)$$

raises R^2 to 0.7781. But a significant amount of curvilinearity was evident, chiefly in log A.U. It turned out that the data in this particular sample were adequately fitted by

$\log F = 2.114 - 0.8507\,(\log \text{T.A.} + 1) + 0.1344\,(\log \text{A.U.} - 1)^2$ with $R^2 = 0.7930$.

The authors' 216th point is still aberrant as judged by this new equation, but their first four points are nicely estimated:

	Points						
	1	2	3	4	5	6	216
Observed F.....	6	13	9	11	25	22	888
Estimated F.....	7.0	9.0	7.4	10.6	10.6	9.4	358

Points 5 and 6, while deviating considerably from regression, are by no means as divergent as others among the 209 points fitted. There is no evident reason, therefore, for excluding these six points from the fitting.

It is possible to make some comparisons between the least squares method just described and the graphical fitting presented by Becker and his fellow workers. From their Table 1, the sum of the squares, $(\log F - \log F_m)^2 = 1.766$, was calculated, leading to an estimate of their multiple regression, $R^2 = 0.8578$, greater than the best reported above, 0.7930. While no exact test of significance is available, a rough approximation may be attempted. Nine of the curves drawn by the authors were parabolic in form, but with some obvious divergencies to follow the

data. Perhaps three or four degrees of freedom may be assigned to them; say 33 for the nine. The 10th irregular closed curve (which apparently didn't greatly improve the fitting) can scarcely have required less than five degrees of freedom, making 38 in all. This leads to the following conjectural analysis of variance applied to 209 points:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Total deviations of logarithms	208	12.421	.....
Constants in least squares regression	2	9.850	4.925
Deviations from least squares regression	206	2.571	0.0125
Deviations from graphical fitting	170	1.766	0.0104
Constants in graphical fitting	38	10.655	0.280
Excess of constants, graphical over least squares	36	0.805	0.0224

$F = 0.0224/0.0104 = 2.15 \qquad F_{.01} = 1.75$

If this procedure be reasonable, it becomes clear that the graphical fitting is the more effective in respect of both average deviation from regression and total reduction in sum of squares. But this advantage is gained at the expense of reduction in sum of squares per constant fitted. Here the number of constants used in the graphical fitting exacts no penalty because of the many degrees of freedom available.

THE ESTIMATION OF A.P.

The ultimate objective of Becker and his collaborators was to estimate the atabrine concentration in the blood plasma by means of simple field measurements of urine characteristics. The most obvious relation to try was the correlation between the concentrations of atabrine in the plasma and in the urine. The report was that, "when plasma levels (A.P.) were plotted on the ordinates and corresponding urinary levels (A.U.) on the abscissas in the manner of a correlation chart, the widely scattered points demonstrated the lack of any considerable degree of correlation." This finding is not surprising since the correlation is only 0.5, the regression curved, and the variance heterogeneous. Since ratios were used effectively throughout the authors' investigation, it seemed worth while to the present writers to examine the correlation between the logarithms of A.P. and A.U. These logarithms were found to follow the normal bivariate distribution fairly closely with $r^2 = 0.5682$. This is comparable with the authors' final correlation between computed and observed plasma atabrine levels, $R^2 = 0.5722$. Had these investigators been fortunate enough to have observed this relation among the logarithms, they could have attained their objective by the use of the simple graphical computing device illustrated in Figure 1, where field determinations of urinary atabrine (A.U.) may be located on the upper scale and corresponding estimates of plasma atabrine (A.P.) read on the lower. The calculation

of C.A.P. would have been avoided, and the resulting estimates of A.P. would have been practically as accurate, on the average, as those designated as C.A.P.

Other investigators had used a relation that suggested

$$\text{A.P.} = \text{k A.U./T.A.}$$

The authors tried plotting the ratio, A.U./T.A., against A.P. but were disappointed. It turns out that the logarithms are linearly related,

$$\log \text{A.P.} = 0.429 \log \text{A.U.} - 0.212 \log \text{T.A.} - 0.104,$$

with $R^2 = 0.6393$. From internal evidence, this would seem to be the best fitting equation that can be found: there is no regular (curved) deviation

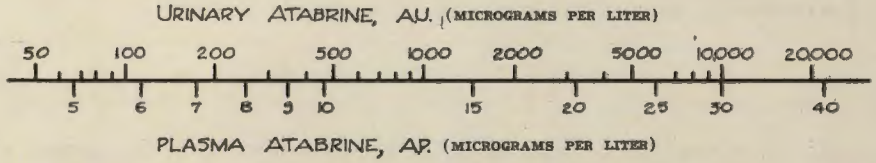

FIG. 1.—Scales for estimating Plasma Atabrine (A.P.) from Urinary Atabrine (A.U.). The equation is $\log \text{A.P.} = 0.3595 \log \text{A.U.} + 0.0398$.

from it, and the variance of the deviations is reasonably homogeneous. Use of this equation might be forbidding to those not accustomed to logarithms, especially if they are in the field, but the graphical solution in Figure 2 is easier than the combination of chart reference with computation which was furnished by the authors. The graphical calculation illustrated is for case No. 5, which the authors placed in Group A_1 without attempting to estimate the plasma atabrine. The deviation from regression for this case is $4 - 8 = -4$, substantially less than the median deviation which is about 6.5. In fact, none of the members of this group show as much as average fluctuation from the regression.

Retransformed to the original units, the equation,

$$\text{A.P.} = 0.787 \frac{\text{A.U.}^{0.429}}{\text{T.A.}^{0.212}},$$

indicates why the authors failed to find a linear relation between A.P. and A.U./T.A. The two independent variables are not equally effective in estimating A.P., the standard partial regression coefficients of log A.P. on log A.U. and log T.A. being, respectively, 0.900 and —0.304.

It is interesting to reconsider case No. 216 which was treated as aberrant because of its large deviation from estimated F. Its A.U. = 3,220 and T.A. = 0.3 applied to Figure 2 give an estimated value of A.P. = 33 as compared to the observed value, 12. The deviation, —21, is large; but it is exceeded in magnitude by the deviation for case No. 164, which is 28, and is not much greater than the deviation for case No. 192, which is

18. Thus, if the authors had not been diverted by their discovery of F, they would probably not have rejected any of their 216 sets of observations.

The worth of any empirical curve fitting must be judged ultimately by success in predicting behavior in other samples from the same population. In an addendum the authors note the fact that a hundred additional determinations had been available for study. These would have comprised a valuable means for testing the results which we have described, but Dr. Becker tells us that they were war casualties.

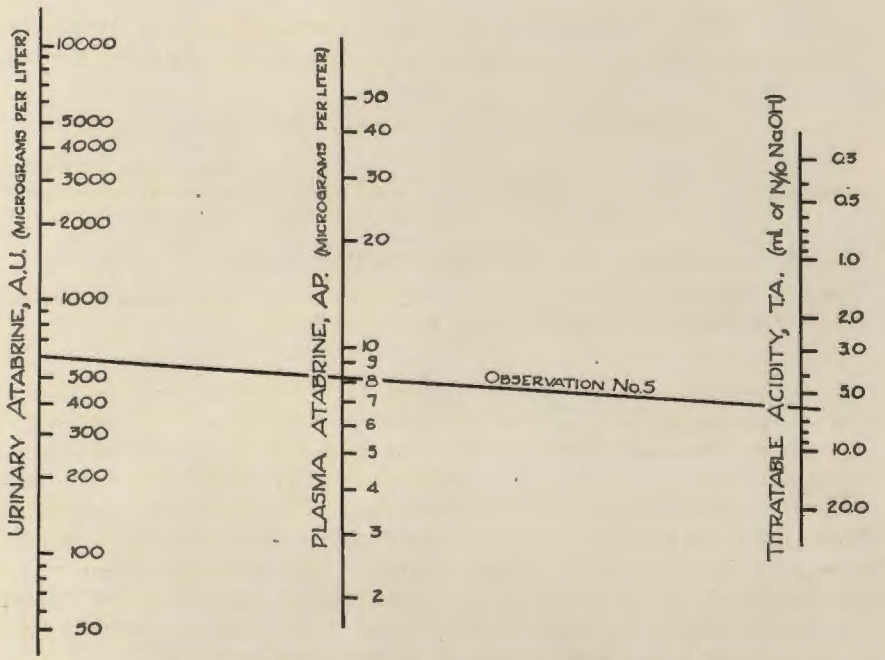

FIG. 2.—Alignment chart for estimating Plasma Atabrine (A.P.) from determinations of Urinary Atabrine (A.U.) and Titratable Acidity (T.A.) of the urine. The oblique line illustrates the estimation of A.P. = 8 from the observed values for case No. 5; A.U. = 600, T.A. = 6.

The answer to the question of the title seems to be that curve fitting is, in large measure, an art; but a modicum of science may not be amiss.

SUMMARY

Becker, Carter, Burks, and Kaleita were successful in fitting the function F to the data described in the July, 1946, number of this *Journal*. The evidence is that no continuous surface with a moderate number of degrees of freedom would fit the data so closely as did their graphical curves. There is some indication, however, that the population regression

may be almost as well described by a simple logarithmic function as by their graph.

As compared with the authors' method, the estimation of plasma atabrine (A.P.) can be done as effectively and more conveniently by the use of either of two graphical computing devices which are presented in Figures 1 and 2.

OXIDATION OF POLYHYDRIC ALCOHOLS BY *ACETOBACTER SUBOXYDANS*[1]

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From a physicochemical viewpoint, fermentation is a special case of catalysis, or rather autocatalysis, in heterogeneous system. The catalysts, the enzymes, are manufactured during the course of the reaction through the growth of the microorganism involved. Deliberate use of microorganisms as a series of catalysts, of graded oxidizing and reducing tendencies, may be illustrated by the oxidation of polyhydric alcohols by species of the genus *Acetobacter*. The reaction is one of dehydrogenation of a secondary alcohol group and thus furnishes a simple model from the viewpoint of physical chemistry.

As early as 1852, Pelouze (27) subjected the juice of the mountain ash berry to spontaneous fermentation in open vessels. After fourteen months, he obtained from the fermented liquors a crystalline material with the molecular formula $C_6H_{12}O_6$; the compound was levorotatory and reduced Fehling's solution. In 1896, Bertrand (1) proved this compound, sorbose, to be formed by bacterial action upon the polyhydric alcohol sorbitol. This "sorbose bacterium" is now known as *Acetobacter xylinum*. Bertrand (1,2,3,4,5,6,7,8) reported the oxidation of eight different sugar alcohols by the organism to give the corresponding ketose sugars. The oxidative action proved to be highly specific. Only the secondary alcohol group next to the primary alcohol group on one end of these polyhydric alcohols was found to be oxidized. Moreover, Bertrand (2) stated that the contiguous secondary alcohol group must be in the *cis* position. He foresaw many uses of this reaction in carbohydrate chemistry.

Subsequently it was found that several species of the genus *Acetobacter* promote similar oxidations. An excellent survey of the biochemical activities of the acetic acid bacteria has been given by Butlin (10). Kluyver and DeLeeuw (25) isolated *Acetobacter suboxydans*, which has been most widely used in recent years due to its superior cultural characteristics and oxidative behavior. As will be shown later, it is even more specific in its reactions than is *Acetobacter xylinum*.

In our laboratories, the main emphasis has been placed on developing conditions for obtaining maximum yields of the keto-compounds and methods for their isolation and purification. The first part of this paper deals with this phase of the problem, to be followed by a general dis-

[1] Presented before the Division of Sugar Chemistry and Technology at the 110th Meeting of the American Chemical Society, Chicago, Sept. 11, 1946.

cussion of stereochemical considerations of special interest to carbohydrate chemists.

SORBITOL TO SORBOSE

In 1935 a fellowship was established in our laboratories, by two pharmaceutical concerns to explore the possibility of the microbial synthesis of ascorbic acid or Vitamin C. The proposed steps involved the oxidation of sorbitol to sorbose and the conversion of the latter to ascorbic acid. The present discussion deals only with the conversion of sorbitol to sorbose. Bertrand and others had prepared sorbose by the action of *Acetobacter xylinum* on media containing only 3 per cent to 6 per cent of sorbitol. The yields of sorbose were relatively low and fermentation periods of several weeks were required.

Four species of the genus *Acetobacter* were tested in our laboratories on a medium containing 0.5 per cent yeast extract (Difco) and 15 per cent sorbitol, a much higher concentration of substrate than had been previously employed. The relative yields of sorbose are shown in Table 1. This relative order proved identical to that given by Kluyver and De-

TABLE 1

RELATIVE YIELDS OF SORBOSE BY FOUR SPECIES OF THE GENUS *Acetobacter*

Organism	Relative Yield of Sorbose
Acetobacter suboxydans	100
Acetobacter aceti	85
Acetobacter xylinum	75
Acetobacter peroxydans	5

Leeuw (25) for the increasing oxidizing tendency of these species of *Acetobacter*. The *Acetobacter peroxydans* proved so active as to give relatively large quantities of carbon dioxide and water, and little sorbose. It is evident that *Acetobacter suboxydans* is the best organism for the reaction under consideration and it was employed in all of our studies on the oxidation of polyhydric alcohols. This organism has a further advantage over *A. aceti* and *A. xylinum* in that it does not grow in a sticky, cellulosic mat, but forms a friable growth permitting easy removal and separation from the fermented medium.

Preliminary experiments with *A. suboxydans* in the oxidation of sorbitol showed the optimum temperature to be from 25° to 30° C.; pH from 5.1 to 6.8; and that at least 0.5 per cent of yeast extract must be present. Later it was found that these conditions also held for the oxidation of the other polyhydric alcohols studied. Hence, the following conditions were maintained in all subsequent work: 28° C., pH = 6.1, and 0.5 per cent yeast extract (Difco). The pH of 6.1 was most convenient since all media were at or near this value when made up and sterilized.

It was found that disturbing the flasks to take samples materially reduced the yields of oxidation product. Hence, each datum presented

represents the analysis of a separate flask. Proper aeration of the culture speeds up the rate of oxidation and obviates the necessity of care in not disturbing the surface growth. However, difficulties in obtaining uniformity of aeration and duplication of equipment precluded its use in running series on a large number of small flasks.

Using a medium containing 10 per cent sorbitol, data were obtained on the effect of the surface-volume ratio on the yields of sorbose. Typical data are shown in Table 2. It is evident that both the reaction rate and

TABLE 2

EFFECT OF SURFACE-VOLUME RATIO UPON THE PER CENT YIELD OF SORBOSE BY THE ACTION OF *Acetobacter suboxydans*

(10 Per Cent Sorbitol Medium)

Days	Surface-Volume Ratio ($cm.^2$ per cc. medium)					
	2.360	1.195	0.589	0.345	0.200	0.119
1.5	78	67	41	28	15	10
2.5	84	82	58	42	26	14
3.5	84	84	77	56	35	21
4.5	85	84	80	63	39	24
5.5	87	84	81	72	45	30
6.5	87	84	82	80	48	.....
7.5	89	84	82	80	53	31

final yield increase with increase in surface-volume ratio. Since similar relations held for the other polyhydric alcohols studied, a surface-volume ratio of about 1.195 was used in all subsequent experiments.

In Table 3 are given typical data showing the effect of the concentration of sorbitol on the yield of sorbose. It is evident that while the rate of oxidation decreases with increasing concentration of substrate, the final yield of sorbose is practically independent of the sorbitol concentration up to and including 35 per cent; there was a marked drop in final

TABLE 3

EFFECT OF CONCENTRATION OF SORBITOL UPON THE PER CENT YIELD OF SORBOSE BY *Acetobacter suboxydans*

Percentage Sorbitol	Days				
	2	3	7	11	14
10	80	83	84	.....	.....
15	72	.....	86	85	84
20	.....	64	80	83	83
25	.....	49	73	82	85
30	.....	34	62	76	78
35	16	.....	67	81	80
40	3	.....	9	14	14
45	2	.....	3	4	4
50	2	.....	.....	2	2

yield at 40 per cent. With 35 per cent sorbitol the concentration of sorbose reached the high value of 28 grams per 100 ml. of fermented medium.

The employment of these high concentrations of sorbitol affords an easy method for the large scale production of sorbose with the handling of minimum volumes of liquids. The sorbose is readily recovered by filtering the fermented medium and evaporating it to the required volume for crystallization. Details of the initial experimental work were published by Fulmer, Dunning, Guymon, and Underkofler (17).

The rapid development of the process may be shown by the following sequence. At the time of initiation of the project one chemical house offered five grams of sorbose "price quoted on request." Within three months we were preparing sorbose in 15 pound lots, production being limited only by requirements and equipment. Colleagues in the Agricultural By-Products Laboratory, at Ames, studied the fermentation in their special rotating drum, developed for the production of gluconic acid by submerged growth of *Aspergillus niger,* under aeration. Soon they were preparing sorbose in 200 pound lots. Their results on the pilot-plant scale production of sorbose were published by Wells, Stubbs, Lockwood, and Roe (41) just two years following the initiation of our project. Within a short time this fermentation formed the first step in the industrial production of synthetic ascorbic acid.

MANNITOL TO LEVULOSE

In the conversion of mannitol to levulose by *Acetobacter suboxydans* it was found by Fulmer, Dunning, and Underkofler (18) that while the rate of oxidation decreased with increasing concentration of substrate, the final yield was practically independent of the concentration of mannitol up to and including 25 per cent; there was a marked decrease in final yield at 30 per cent. With 25 per cent mannitol the yield of levulose was better than 90 per cent of theory after seven days. Evidently both sorbitol and mannitol are efficiently oxidized at relatively high concentrations.

GLYCEROL TO DIHYDROXYACETONE

In the conversion of glycerol to dihydroxyacetone the concentration of the substrate should not exceed 6 per cent for maximum yields with surface cultures. This is in contrast to sorbitol and mannitol which are efficiently oxidized at concentrations of at least 25 per cent. Incubation beyond seven days did not give increased yields, at which period the yield of dihydroxyacetone was 90 per cent of theory or better. Methods were developed for the isolation of the dihydroxyacetone by which 80 per cent of the product was obtained in the crystalline form. The details were published by Underkofler and Fulmer (36).

This work made dihydroxyacetone, a highly important ketotriose, available to American chemists and biochemists. Subsequent to the publication mentioned, experience has led to modifications which simplify the preparation, recovery, and purification of the dihydroxyacetone.

The medium employed contains 5 g. of pure glycerol, 0.5 g. of Difco

yeast extract, and 0.25 g. of monopotassium phosphate per 100 ml. It is distributed in flasks which are plugged with cotton and sterilized by heating at 15 pounds steam pressure for 15 to 30 minutes. For surface culturing 300 ml. of medium are used in each 2-liter Erlenmeyer flask. For submerged culturing any convenient size of flask may be employed which is about two-thirds filled with the medium and equipped for aeration with sterile air through efficient air dispersers such as those made of alundum. The sterile medium is inoculated with 2 to 5 per cent of a 24-hour culture of *Acetobacter suboxydans* grown on a medium of the same composition and then incubated at 28° C. Surface cultures are incubated for seven to ten days without disturbing the flasks and submerged cultures are incubated for three to seven days with continuous aeration at the rate of at least 200 ml. of air per liter per minute. If desired the completion of the fermentation may be determined by periodic reducing sugar analyses, the incubation being interrupted when the maximum reducing value has been reached.

The fermented liquid is worked up in the manner described by Underkofler and Fulmer (36) to the point of crystallizing the sirup. The sirup is placed in a beaker over sulfuric acid in a vacuum desiccator which is evacuated with a Hyvac pump until the mixture boils. The vacuum is renewed at least twice each day for two or three days, each time to the point where there is an actual boiling of the sirup. After this period the vacuum is released and the thick sirup seeded with a few crystals of dihydroxyacetone and the vacuum again applied. In two or three days after seeding, the mass crystallizes to a solid, glassy consistency. The beaker is placed in an ice bath and a half-volume of cold absolute alcohol added. The mass is worked with a spatula until it can be transferred to a cold, dry mortar and is there triturated to a smooth paste. The material is filtered by suction, the solid again triturated with a small amount of cold absolute alcohol and again filtered by suction. The product is thoroughly dried over calcium chloride in a vacuum desiccator. The crude dihydroxyacetone so obtained is slightly colored and is stored in a tightly stoppered dark glass bottle in the refrigerator, being further purified for use as required. Additional quantities of the compound may be obtained from the mother liquor and wash liquid by working up in the same manner as before.

The crude dihydroxyacetone is recrystallized from hot absolute alcohol, taking care to prevent access of moisture since traces of water prevent satisfactory crystallization. The crude material is dissolved in boiling absolute alcohol. If the solution is colored, it is decolorized (while hot) with Norite and filtered by suction. Most of the alcohol is distilled off by using a wire gauze and open flame under the flask. The residual small volume of material is cooled thoroughly in a well-stoppered flask, seeded with a few crystals of pure dihydroxyacetone and shaken. After crystallization has started, the stoppered flask is placed in the refrigerator for a few hours, with occasional shaking. The solid is filtered on a cold filter by suction and washed with cold absolute alcohol until it is pure

white. It is then dried over calcium chloride in a vacuum desiccator. Additional amounts of the pure crystalline dihydroxyacetone can usually be obtained by distilling the alcohol from the combined mother liquors and wash liquids and following the same procedure as before. The pure dihydroxyacetone should be stored in a well-stoppered dark glass bottle in a cool place, preferably in a refrigerator.

The pure dihydroxyacetone prepared as above is the usual bimolecular alpha-modification. The crystals are colorless, prismatic plates, very soluble in water, sparingly soluble in organic solvents, and melt indefinitely at 68°–80° C. The unimolecular beta-modification may be obtained by the method of Fischer and Mildbrand (15) by distilling the dihydroxyacetone under high vacuum.

It is preferable to purify and recrystallize the dihydroxyacetone shortly before use, since on standing it spontaneously condenses through loss of water into a pasty mass containing substances of higher molecular weight and of limited solubility in water. This change occurs much more slowly when the purified material is stored in the dark in the refrigerator.

The best method of purifying dihydroxyacetone which has undergone this decomposition appears to be that of Reeves and Renbom (31) as follows: Suspend 50 g. of the pasty material in 100 ml. of pure acetone and shake continuously for 24 hours at room temperature (best below 25° C.). Filter with suction, wash with acetone and dry thoroughly over calcium chloride in a vacuum desiccator. After the acetone treatment and washing, the material may be recrystallized immediately from absolute alcohol as described above.

ERYTHRITOL TO ERYTHRULOSE

Whistler and Underkofler (42) studied the oxidation of *meso*-erythritol to L-erythrulose and found the concentration of substrate should not exceed 4.5 per cent. Under optimum conditions the yield of L-erythrulose was practically quantitative in seven days. Methods were developed by which 87 per cent of the erythrulose was recovered from the fermented medium as a colorless sirup.

OXIDATION OF *meso*-INOSITOL

Studies were made on the oxidation of the cyclic polyhydric alcohol *meso*-inositol (or *i*-inositol) and reported by Dunning, Fulmer, Guymon, and Underkofler (13) and Dunning, Fulmer, and Underkofler (14). Since this compound contains only secondary alcohol groups it offered an interesting test of the relation of configuration to the action of the organism. Moreover, this type of oxidation, if successful, would permit the preparation of cyclic ketones not heretofore available. *meso*-Inositol is also of biological interest because of its wide distribution in nature as a component of phytin, its identity with the yeast growth stimulant Bios I and the fact that it is also classed as a vitamin.

The development of the medium for the oxidation of inositol furnished an especially interesting problem. The culture could not be carried be-

yond the fifth transfer on an inositol-yeast extract medium. However, the presence of as little as 0.025 to 0.05 per cent of sorbitol permitted continuous subculture and a high conversion of the inositol to a reducing compound. At first it was thought that some factor was formed by the action of the organism on sorbitol favoring the growth and oxidative action on the inositol. Further studies, however, showed that the sorbitol merely served as a substrate allowing the growth of the organism with accompanying production of enzymes capable of oxidizing the inositol. That is, *A. suboxydans* can assimilate and oxidize sorbitol; it cannot assimilate but can oxidize the *meso*-inositol. Low concentrations of other assimilable substances such as glycerol, erythritol, dextrose, and mannitol were found to serve the same purpose as did sorbitol. However, the presence of such assimilable substrates did not permit the oxidation of dulcitol or of L-rhamnitol, compounds, not attacked by the organism because of their unfavorable configurations.

Since methods for preparation, recovery, and purification of the oxidation product of *meso*-inositol have not been previously published, they are now presented.

The medium employed contains 3 g. of *meso*-inositol, 0.05 g. of sorbitol, and 0.5 g. of Difco yeast extract per 100 ml. It is distributed in flasks, sterilized, inoculated, and fermented in the same manner as described above for the preparation of dihydroxyacetone. Upon completion of the fermentation a sufficient amount of lead acetate solution is added to give a final concentration of about 0.75 per cent. Then 10 g. each of Norite and diatomaceous earth per liter are added, the mixture is thoroughly shaken and filtered by suction. Excess lead is removed from the filtrate by treating with hydrogen sulfide and the solution filtered with suction. The clear filtrate is concentrated by vacuum distillation to about one-half the original volume, and again treated with hydrogen sulfide. After suction filtration the evaporation is continued *in vacuo* until crystallization begins. The material is then placed in the refrigerator overnight for completion of precipitation, the solid finally being filtered off and washed with 50 per cent alcohol. The yield is as much as 75 g. of product per 100 g. of *meso*-inositol originally present in the fermentation medium.

The crude product is only slightly soluble in cold water but dissolves in boiling water to give a brown colored solution. Norite is added, the mixture boiled, and then filtered to obtain a clear, colorless solution. The solution is evaporated *in vacuo* to the point of crystallization, then alcohol is stirred in until a concentration of 60 to 70 per cent alcohol is reached. The mixture is allowed to remain in the refrigerator overnight to ensure complete crystallization and is then filtered. Purification consists in recrystallizing at least once more from water by the above procedure and then two or three times by precipitation with alcohol. The latter is accomplished by dissolving the solid in a minimum quantity of boiling water, and then slowly adding alcohol while the mixture is maintained at the boiling temperature until a concentration of 60 to 70 per cent alcohol is reached. The solution is then cooled and the solid recovered by suction filtration.

The physical properties of the compound depend upon the method employed for the final crystallization. When crystallized by precipitation with alcohol from the boiling aqueous solution, a macro-crystalline product is obtained which is soluble in water. When crystallized from water by evaporation and cooling, a micro-crystalline product is obtained which is difficultly soluble in water. The melting point of a given preparation is quite sharp, but may be varied from 184° to 195° by altering the method of crystallization.

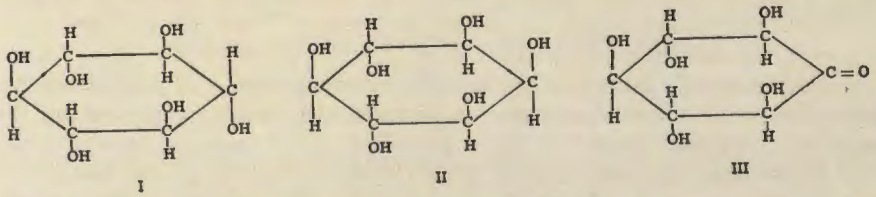
Regardless of the method of crystallization the compound was found to be soluble in warm, dilute alkali, and insoluble in dilute acids, concentrated sulfuric acid, and organic solvents. The physical and chemical characteristics of the compound showed it to be a keto-inositol. To assist in characterizing the compound the amorphous phenylhydrazone and dinitrophenylhydrazone, and the crystalline acetyl derivative were prepared and carefully purified. In Table 4 are given the melting points and analytical data on these derivatives, the values being compared with those given by Posternak (28,29) for two monoketo-*meso*-inositols or "inososes." The first of these, designated as *epi*-inosose, he prepared by chemical oxidation of *meso*-inositol, and the second, designated as scyllo-*meso*-inosose, by oxidation of *meso*-inositol with *A. suboxydans*. Kluyver and Boezaardt (24) had previously reported the isolation of the latter compound upon oxidizing *meso*-inositol with this organism, and believed it to be identical with the inosose previously prepared by Posternak by chemical oxidation. Posternak (29) found the properties of the derivatives of the two inososes to be different. Posternak (30) showed the configuration of the inosose obtained by oxidizing *meso*-inositol with *A. suboxydans* to be related to scyllitol (I) as well as to *meso*-inositol (II),

TABLE 4

COMPARISON OF INOSOSES WITH THE FERMENTATION KETO-INOSITOL OF DUNNING, FULMER, AND UNDERKOFLER

Posternak's (28) chemical *epi*-inosose:	
Recrystallized compound, M.P. 198–200° C.	
Phenylhydrazone, M.P. 220–222° C.	
2,4-Dinitrophenylhydrazone, M.P. 270° C.; nitrogen calculated for monoketo-inositol 12.2 per cent.	
Acetyl derivative (penta-acetate), M.P. 106–108° C.	
Biochemical scyllo-*meso*-inosose (24, 29):	
Recrystallized compound, M.P. 200–202° C.	
Phenylhydrazone, M.P. 184° C.	
Acetyl derivative (penta-acetate), 2 crystalline forms, M.P. 211°; 147° C.	
Iodine absorbed in alkaline medium by Willstätter-Schudel (43) procedure, 1.87 equivalents.	
Keto-inositol of Dunning, Fulmer, and Underkofler:	
Recrystallized compound, M.P. 184–195° C.	
Phenylhydrazone, M.P. 218° C.	
2,4-Dinitrophenylhydrazone, M.P. 193–195° C.; nitrogen calculated for diketo-inositol 20.9 per cent, found 21.0 per cent.	
Acetyl derivative (tetra-acetate), M.P. 114° C.; acetyl calculated for tetra-acetate of diketo-inositol 50.0 per cent, found 50.4 per cent.	
Iodine absorbed in alkaline medium by Willstätter-Schudel (43) procedure, 3.85 equivalents.	

which fixed the position of the carbonyl group in the compound, giving the configurational formula (III) for the scyllo-*meso*-inosose.



That is, under the conditions used by Posternak, *A. suboxydans* preferentially oxidizes the middle one of the three *cis* hydroxyl groups of *meso*-inositol.

The data of Table 4 indicate clearly that the product obtained by oxidizing *meso*-inositol with *A. suboxydans* during our investigations was not identical with either of the inososes studied in detail by Posternak. The fact that our product was quite different from the compound later secured by Kluyver and Boezaardt (24) and by Posternak (29) with the same organism is difficult to explain. Private communications from workers in several other laboratories in this country have indicated that on oxidizing *meso*-inositol with *A. suboxydans*, certain of these have obtained products resembling in properties the compound studied by Posternak (29), whereas others have obtained products with properties corresponding to the compound obtained in our laboratories. Differences in the strain of *A. suboxydans* employed, cultural conditions, or even conceivably in the source of the inositol, may influence the course of the reaction. Further work will be required to decide this question.

The data of Table 4 indicate our compound to be a diketo-*meso*-inositol. The complexity of further identification is indicated by the fact that fourteen diketo-*meso*-inositols are possible.

Recently Chargaff and Magasanik (11) published a note on the oxidation of stereoisomers of the inositol group by *A. suboxydans*. Through isolation as the phenylosazones, these workers demonstrated the formation of α-diketo derivatives of inositol by the action of the organism on *l*-inositol and *d*-inositol, and through isolation as the phenylhydrazone, the formation of a monoketo derivative of inositol from *epi*-inositol.

Comparative studies were made on the effects of *meso*-inositol and of our keto-inositol on the growth of yeast. It was necessary to exclude the possibility that the compound might be contaminated with yeast extract which would furnish yeast growth factors. Therefore, the phenylhydrazone derivative was prepared in dilute acid solution and the product recrystallized. The keto-compound was recovered by hydrolysis with benzaldehyde and a little benzoic acid according to the method of Posternak (28,29). The purified compound was found capable of stimulating yeast growth, alone and in a variety of combinations with nine known stimulants, with three types of *Saccharomyces cerevisiae*. The keto-inositol served essentially the same role as *meso*-inositol but in the

majority of cases showed a greater initial growth stimulation. It is interesting to speculate as to the possibility that the interchangeability of the two compounds means that they form a reversible oxidation-reduction system.

2,3-BUTANEDIOL TO ACETYLMETHYLCARBINOL

Only recently have the three stereoisomeric forms of 2,3-butanediol become available by other than laborious synthetic methods. As will be seen below, means have now been found for their preparation and isolation by biological means.

In our laboratories methods were developed by Fulmer, Christensen, and Kendall (16) giving nearly theoretical yields of 2,3-butanediol by the action of *Aerobacter aerogenes* on sucrose. The glycol so produced is slightly dextro-rotatory $[\alpha]_D^{30} = +1.0$. This glycol will be referred to subsequently as "Aerobacter glycol." Freezing point data indicated the glycol to consist of about 90 per cent of the *meso*-form.

On the other hand, Ward, Pettijohn, Lockwood, and Coghill (40) reported that *Aerobacillus polymyxa* ferments starchy materials with the production of levo-rotatory 2,3-butanediol, $[\alpha]_D^{30} = -13.0$. During the war period both types of fermentation were carried through the pilot plant scale in a number of different laboratories.

Fulmer, Underkofler, and Bantz (19) found that the "Aerobacter glycol," when acted upon by *A. suboxydans*, gave a yield of 90 per cent of acetylmethylcarbinol. As was the case with *meso*-inositol, the culture could not be carried beyond the fifth transfer on a glycol-yeast extract medium. Similarly, the addition of a very low concentration of an assimilable substrate permitted continuous subculture and rapid oxidation of the glycol. The residual glycol, recovered from the fermented medium, was dextro-rotatory $[\alpha]_D^{30} = +10.15$. Hence, the organism attacked the *meso*-2,3-butanediol but did not oxidize the dextro-rotatory form. This high specificity furnishes a method for separating the two forms. The *meso*-form can best be isolated from "Aerobacter glycol" by fractional crystallization from isopropyl ether by the method of Wilson and Lucas (44).

Underkofler, Fulmer, Bantz, and Kooi (37) found that the levo-rotatory 2,3-butanediol was oxidized almost quantitatively to acetylmethylcarbinol by *A. suboxydans*. That is, the organism oxidizes the *meso*- and levo-rotatory glycols but does not attack the dextro-rotatory form. By appropriate use of *Aerobacter aerogenes*, *Aerobacillus polymyxa*, and *Acetobacter suboxydans* it is feasible to prepare and isolate the three stereoisomeric 2,3-butanediols.

THE FOUR GROUPS OF POLYHYDRIC ALCOHOLS

The polyhydric alcohols studied in our laboratories fall into four groups with reference to cultural conditions for maximum yields of oxidation products with *Acetobacter suboxydans*. These groups are:

I. Oxidized at high concentrations (25 per cent or above)—sorbitol and D-mannitol.
II. Oxidized at relatively low concentrations—glycerol and *meso*-erythritol.
III. Require additional assimilable substrate for continuous subculture—*meso*-inositol, *meso*-2,3-butanediol and D-(—)-2,3-butanediol.
IV. Not oxidized even in the presence of an additional assimilable substrate—dulcitol, L-rhamnitol and L-(+)-2,3-butanediol.

STEREOCHEMICAL CONSIDERATIONS

The discussion of the stereochemistry of the polyhydric alcohols as related to their oxidation by members of the genus *Acetobacter* may well be considered under three categories: (a) oxidation of the sugar alcohols having more than four carbon atoms; (b) oxidation of erythritol and the glycols; and (c) oxidation of the desoxy sugar alcohols.

OXIDATION OF THE SUGAR ALCOHOLS

Bertrand's work (2,6) led him to the conclusions that *Acetobacter xylinum* oxidizes only those sugar alcohols having the *cis* arrangement of the two secondary alcohol groups adjacent to a primary alcohol group and that only the secondary alcohol group contiguous to the primary alcohol group is oxidized. Subsequent work in several laboratories, employing either *A. xylinum* or *A. suboxydans*, has shown no exception to these rules for sugar alcohols having more than four carbon atoms with a hydroxyl group on each carbon atom.

Data are shown in Table 5 for the oxidation of the pentitols. In this and subsequent tables, *A. suboxydans*, *A. xylinum*, and *A. aceti* are designated as *s*, *x*, and *a*, respectively. A question mark indicates that the alcohol has not been tested. Literature references are given after the designation of organism used. Note in Table 5 that, in accordance with

TABLE 5
OXIDATION OF PENTITOLS

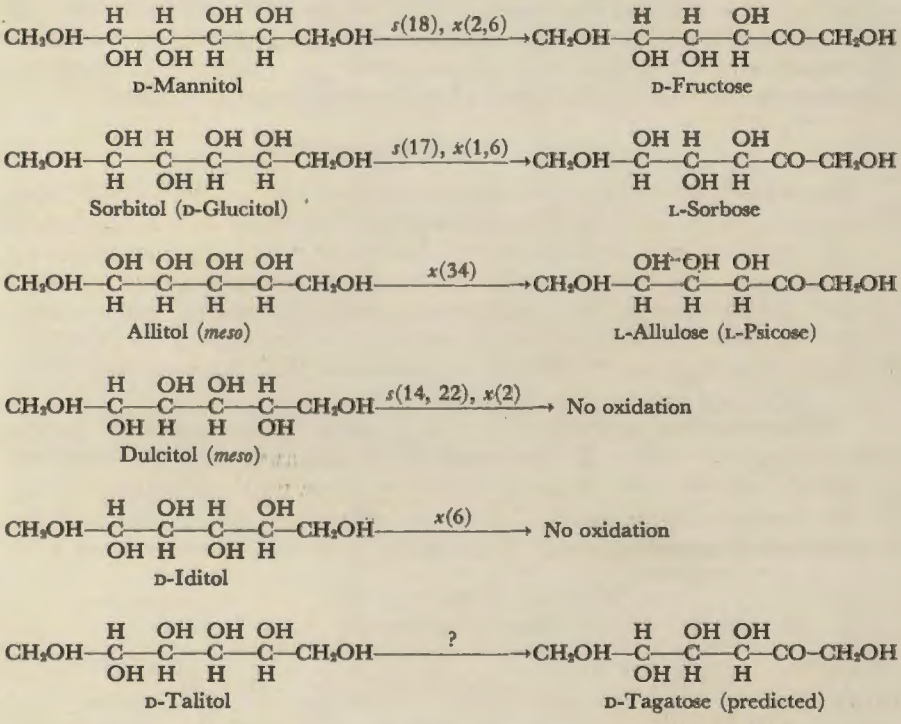
$CH_2OH(CHOH)_3CH_2OH$	*s*(22)	$CH_2OH(CHOH)_2CO{-}CH_2OH$	
D-Arabitol		D-Xylulose	
$CH_2OH(CHOH)_3CH_2OH$	*s*(32)	$CH_2OH(CHOH)_2CO{-}CH_2OH$	
Adonitol (*meso*)		L-Adonulose	
$CH_2OH(CHOH)_3CH_2OH$	*s*(22), *x*(2,6)	No oxidation	
Xylitol (*meso*)			

Bertrand's rule, the two pentitols with the *cis* arrangement of secondary alcohol groups are oxidized. Xylitol, which does not have this configuration is not attacked by the organism. D-Xylulose and L-adonulose have been isolated from the fermented media and conclusively identified.

Table 6 presents data on the oxidation of the hexitols. Only the first five listed have actually been tested and the results conform to Bertrand's rule. The D-fructose, L-sorbose, and L-allulose have been isolated and conclusively identified. Although D-talitol has not been tested, the rule permits the prediction that it should be readily oxidized to D-tagatose.

TABLE 6

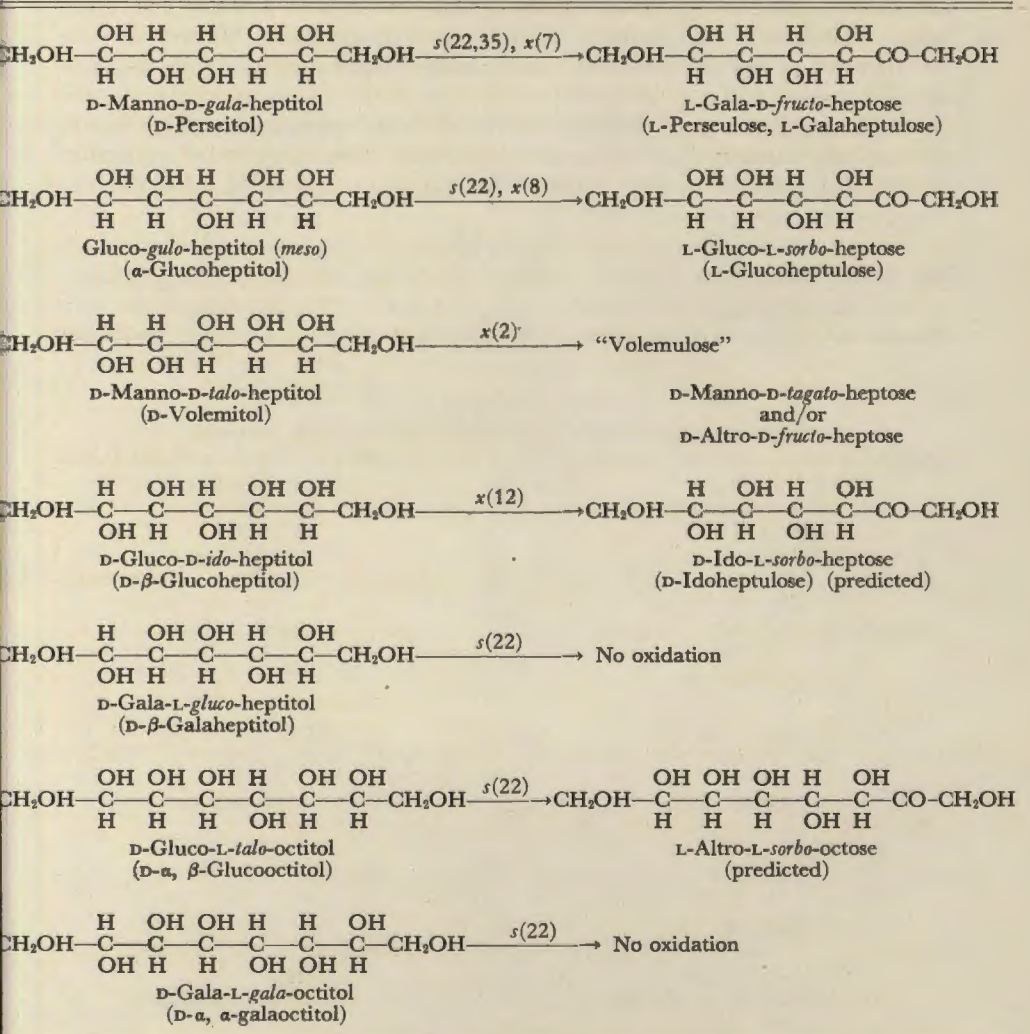
OXIDATION OF HEXITOLS



Information on the oxidation of five heptitols and two octitols which have been tested is given in Table 7. The first four compounds in the table have the *cis* configuration and are oxidized, while the fifth does not possess the favorable configuration and is not oxidized, again confirming Bertrand's rule. The first two heptitols listed have been tested with both *A. xylinum* and *A. suboxydans*. Bertrand (7,8) isolated the ketoses formed by *A. xylinum*. Hann, Tilden, and Hudson (22) were the first to report the oxidation of these compounds by *A. suboxydans*. The L-perseulose was isolated by Tilden (35) and the structure and configuration proven

TABLE 7

OXIDATION OF HEPTITOLS AND OCTITOLS



by Hann and Hudson (21). The oxidation product of gluco-*gulo*-heptitol was not isolated by Hann, Tilden, and Hudson (22), but it can be assumed that the ketose is identical with that isolated and identified by Bertrand as produced by the action of *A. xylinum*. This conclusion is justified by the fact that in all previous cases in which the oxidation products have been identified, the same ketoses have been formed by both organisms. This assumption will be taken for granted in subsequent discussion.

The third and fourth heptitols shown in Table 7 were tested only with *A. xylinum* and the oxidation products were not identified. Since

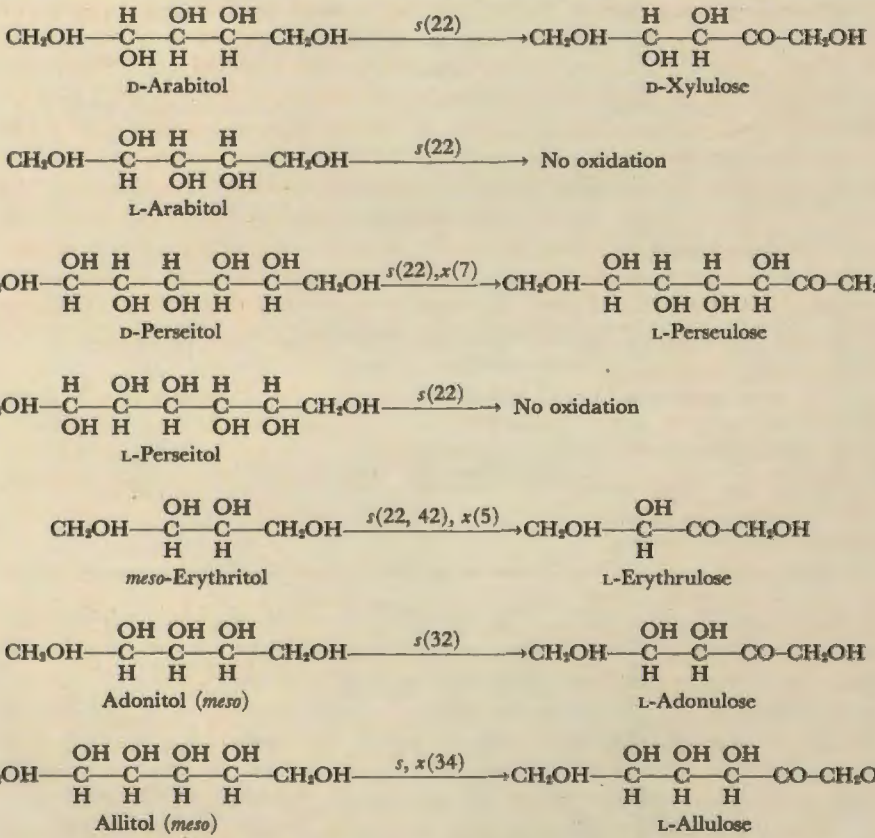
D-manno-D-*talo*-heptitol has the favorable *cis* arrangement on both ends of the molecule, it can be predicted that the reaction would give D-manno-D-*tagato*-heptose or D-altro-D-*fructo*-heptose or a mixture of the two. Bertrand designated the products simply as "volemulose." It can also be predicted that the oxidation of D-gluco-D-*ido*-heptitol would give D-ido-L-*sorbo*-heptose.

The last three polyhydric alcohols of Table 7 were tested by Hann, Tilden, and Hudson (22). The reducing sugar formed from the D-gluco-L-*talo*-octitol was not isolated or identified but the formation of L-altro-L-*sorbo*-octose can be predicted.

Configuration Required for the Oxidation of the Sugar Alcohols Having More than Four Carbon Atoms. Previous discussion has demonstrated conformity to Bertrand's rule that a *cis* arrangement of the two secondary alcohol groups next to the primary alcohol group is required

TABLE 8

D-CONFIGURATION NECESSARY FOR OXIDATION OF SUGAR ALCOHOLS



for the oxidation of the sugar alcohols by members of the genus *Acetobacter*. Hann, Tilden, and Hudson (22) investigated the action of *A. suboxydans* upon a number of sugar alcohols, including two pairs of enantiomorphs; their results with the latter are included in Table 8. It is evident that while D-arabitol and D-perseitol are readily oxidized their enantiomorphs are not attacked. Hann, Tilden and Hudson properly concluded that the organism is so specific that the *cis* pair of secondary alcohol groups must have the D-configuration. The fact that only ketoses of L-configuration are obtained in high yields from the *meso*-sugar alcohols shown in Table 8 confirms this generalization. That *A. suboxydans* is more specific than is *A. xylinum* is further evidenced by the report of Bertrand (5) that L-arabitol is oxidized by the latter organism. Further evidence on the greater specificity of *A. suboxydans* will be presented below. However, no exception has been reported, in which oxidation products have been conclusively identified, to this extraordinarily high specificity of *A. suboxydans*.

OXIDATION OF ERYTHRITOL AND THE GLYCOLS

Data are presented in Table 9 on the oxidation of several polyhydric alcohols having only two secondary alcohol groups. Both *A. xylinum* and *A. suboxydans* produce L-erythrulose from *meso*-erythritol. This compound has the favorable *cis* arrangement of the secondary alcohol groups. *Meso*-2,3-butanediol, which is similar to *meso*-erythritol except that it has two terminal methyl groups is oxidized to L-(+)-acetylmethylcarbinol by *A. suboxydans*. It has been found in our laboratories, however, that the organism also oxidizes the D-(—)-2,3-butanediol but does not attack the L-(+)-2,3-butanediol. That is, with glycols containing the terminal methyl groups, the *cis* arrangement of the secondary alcohol groups is not required. It would be of great interest to test the action of the organism on D- and L-erythritol to determine whether similar results would be obtained with terminal primary alcohol groups.

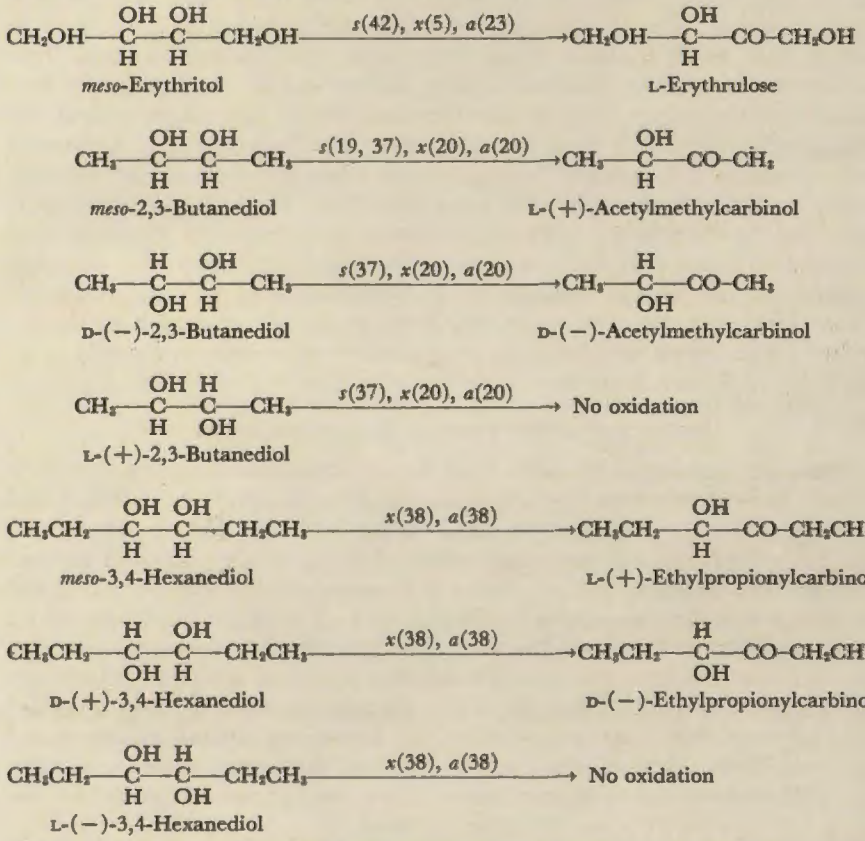
Grivsky (20) confirmed our findings with the 2,3-butanediols using *A. xylinum* and *A. aceti*. However, on prolonged incubation, after complete conversion of the D-2,3-butanediol from a racemic mixture had occurred, the L-diol was slowly oxidized. This was not the case with *A. suboxydans*. This again illustrates the high specificity of the latter organism.

The results shown in Table 9 for the 3,4-hexanediols were obtained by Van Risseghem (38) employing *A. xylinum* and *A. aceti*. With the hexanediols, as with the butanediols, the secondary alcohol group having the D-configuration is preferentially attacked. On the basis of their results, Van Risseghem (38) and Grivsky (20) were able to assign the proper configurations to the 3,4-hexanediols and the 2,3-butanediols. Grivsky's configurations for the butanediols, deduced from microbiological procedure, agree with the configurations assigned to them, on the basis of chemical methods, by Morell and Auernheimer (26).

On the basis of the glycols studied, it may be concluded that secondary

TABLE 9

OXIDATION OF ERYTHRITOL AND GLYCOLS



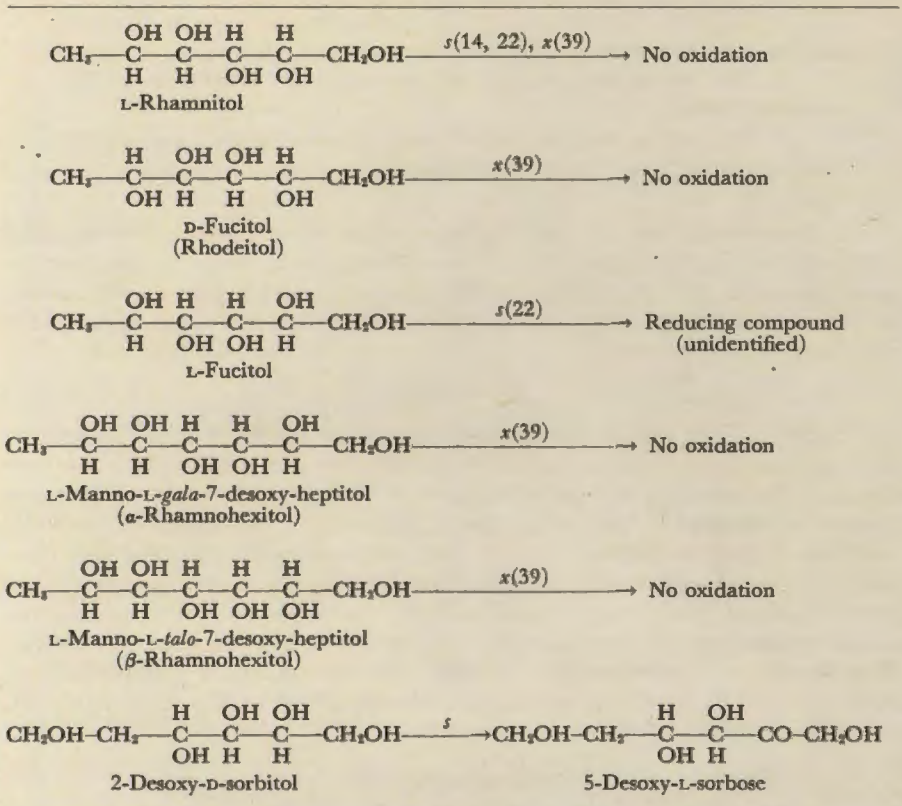
alcohol groups of D-configuration are required for oxidation with *Acetobacter suboxydans* and that those of L-configuration are not attacked. It was previously noted that Hann, Tilden, and Hudson (22) came to the conclusion that Bertrand's rule is followed only in case the *cis* secondary alcohol groups have the D-configuration. With the glycols, only the secondary alcohol group with D-configuration is oxidized but the *cis* arrangement is not required.

OXIDATION OF THE DESOXY SUGAR ALCOHOLS

In Table 10 are shown results of tests reported on the oxidation of several desoxy sugar alcohols by members of the genus *Acetobacter*. All are desoxy in the terminal position except 2-desoxy-D-sorbitol. In an oral paper presented at the 110th Meeting of the American Chemical Society,

TABLE 10

OXIDATION OF DESOXY SUGAR ALCOHOLS



Regna reported that this compound was oxidized by *A. suboxydans* to 5-desoxy-L-sorbose which was isolated and characterized. The oxidative reactions with the desoxy sugar alcohols follow Bertrand's rule of necessary *cis* secondary alcohol groups as well as the required D-configuration except in the case of L-fucitol which apparently breaks both rules. Hann, Tilden, and Hudson (22) found that *A. suboxydans* converted L-fucitol to a reducing substance which was not isolated or identified. We thoroughly agree with these authors in their statement that: "The behavior of L-fucitol must be studied further before deciding what generalization may apply to the alcohols derived from the methylose sugars."

SUMMARY OF STEREOCHEMICAL RELATIONSHIP

Stereochemical relations have been presented for the oxidation of 31 polyhydric alcohols by members of the genus *Acetobacter*. With possible exception of L-fucitol, the following generalizations can be made with

reference to the specificity of *A. suboxydans* in the oxidation of straight chain sugar alcohols:

1. Only the 2-keto compounds are formed from sugar alcohols having terminal primary alcohol groups.
2. The secondary alcohol group oxidized must possess the D-configuration.
3. The polyhydric alcohols having more than two secondary alcohol groups must have *cis* arrangement as well as D-configuration.

It is evident that *A. suboxydans* furnishes a remarkably specific catalytic tool for the production of keto compounds from the polyhydric alcohols and in the elucidation of the stereochemistry and configuration of these compounds. Only a small part of this field has been adequately explored.

SUMMARY

Species of the genus *Acetobacter*, especially *Acetobacter suboxydans*, oxidize certain polyhydric alcohols to produce ketose sugars. This method enables the production on laboratory or commercial scale of ketoses otherwise obtainable only with great difficulty. Production of sorbose, levulose, dihydroxyacetone, erythrulose, a keto-inositol and acetylmethylcarbinol are reviewed, detailed procedures being given for the laboratory preparation of dihydroxyacetone and the keto-inositol. Stereochemical considerations are presented which lead to the conclusion that *A. suboxydans* furnishes a remarkably specific catalytic tool for production of keto compounds from the polyhydric alcohols and in the elucidation of the configurations of these compounds.

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STUDIES ON OXYGEN-CARRYING COBALT COMPOUNDS

I. OXYGEN-CARRYING METALLO-ORGANIC COMPOUNDS. THEIR USE IN THE MANUFACTURE OF OXYGEN

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Received March 4, 1947

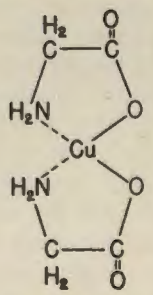
One of the most striking aspects of biochemistry is the use nature has made of small quantities of metals in promoting and controlling the various complicated chemical processes of life. Hemoglobin, utilizing iron in the transport of oxygen by the blood of the mammals, and chlorophyll, utilizing magnesium in the reduction of carbon dioxide by the plants, are the more widely appreciated of these natural, metal-organic materials, but equally intriguing are the copper, manganese, and vanadium oxygen-carriers in the blood of the lower animals and the metal-containing enzymes governing certain biological, oxidation-reduction processes. The well-being of many plants and animals is dependent on the proper functioning of these metallo-organic compounds and on the availability of minute amounts of other metals, particularly zinc and cobalt, whose action is exerted through coordination compounds with protein. An abundant literature already exists describing the effects of deficiencies of these metals, but only a start has been made in elucidating the chemistry of the metallo-organic compounds involved.

The linkage of the metal to the organic molecule in these compounds is through the functional groups of the organic molecules, no case of a direct linkage of carbon to metal being known. The functional group may be an acidic group, in which case the metal replaces a hydrogen atom, or it may be a basic group, to which the metal is attached by a so-called secondary or coordinate valence. When the functional group involved is an acidic group the bond may be ionic or non-ionic in character, that is, the metal may be split off as an ion or held closely to the organic group in a non-ionic or covalent form. The attachment to a basic group is non-ionic in character. In the usual case more than one functional group is present in the organic molecule and a combination of both types of valences is involved. Frequently there is also involved the formation of rings or cages in which the metal is implicated in the ring or cage structure. Such ring structures often possess extraordinary thermal stability; the compounds usually have colors departing widely from the customary colors of the metal salts, and they frequently are soluble in non-polar solvents and insoluble in water.

The composition and structure of the numerous compounds of ammonia and the metals, particularly of cobalt and platinum, puzzled chemists for a century. It was the elucidation of the nature of these

compounds which has supplied the tools for attacking the problems of the naturally occurring metallo-organic compounds. In 1893 the Swiss chemist Alfred Werner devised the coordination theory which explained the composition of the metal ammoniates on the basis of secondary valence, a new type of valence bond by which apparently saturated compounds were able to unite to form new compounds. Coupling this new concept of valence with a stereochemical explanation of the structure of the compounds, Werner was able to organize the ammoniates into a single system and to correlate an enormous mass of chemical information. Werner's studies culminated in 1912 in the optical resolution of a purely inorganic compound which unequivocally established the coordination theory and secured for its author the Nobel Prize. During the subsequent two decades the coordination theory was expanded and applied to a variety of chemical problems, notably by Paul Pfeiffer in Germany and by G. T. Morgan in England. The coordination theory touched many fields of chemistry but it was particularly fruitful in its application to the dyeing of textiles with metallic mordants, to the discovery of new organic analytical reagents, to leather tanning, and to the study of the stereochemical nature of the metal ions.

That ring formation was involved in the union of metals with functional organic compounds was recognized simultaneously by Bruni and Fornara (1) and by Ley (2) in 1904 while studying the bright blue copper compound of glycine, to which they ascribed the structure

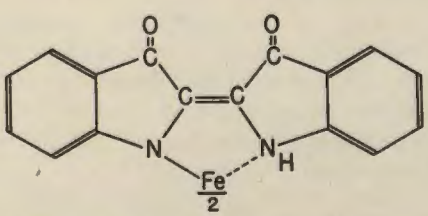


In the following years the conditions necessary for ring formation of this type were pointed out and subsequently numerous compounds, new and old, were shown to possess such a cyclic structure. Drew and Morgan (3) coined the term *chelate ring*, derived from the claw of the lobster and crustaceans, for these cyclic metal-containing compounds. Later Morgan devised the terms *bidentate* and *quadridentate* to distinguish those compounds in which the metal was attached to the organic molecule through two functional organic groups from those in which the attachment was through four groups. The field of the chelate rings has been carefully reviewed in recent years and the reader's attention is directed to a more detailed treatment of this field than is possible here (4).

Although the physiological behavior and the chemical constitution of hemoglobin have been extremely well worked out as a result of the

classical researches of Barcroft (5), Fischer (6), and many others, the nature of the mechanism by which the oxygen becomes attached to the hemoglobin molecule is still uncertain. Indeed, even the nature of the linkage by which heme, the porphyrine part of the molecule, is attached to the protein, or globin, part of the molecule is not known. Fischer, who is largely responsible for the knowledge of the chemical constitution of hemoglobin, has practically nothing to say regarding the attachment of the heme to the globin or the nature of the union of hemoglobin with oxygen. Of the great deal of speculation found in the literature as to the nature of the hemoglobin-oxygen linkage, probably the most reasonable is that of Wahl who has attempted to apply the Werner coordination theory to the problem (7). Reasoning by analogy with certain cobalt-ammonia compounds which are known to absorb oxygen in such a manner as to release it on acidification, Wahl proposed a similar arrangement for hemoglobin. The oxygen enters these cobalt compounds as a bivalent, acidic peroxo group, -O-O-. Similar, simple, peroxo compounds of iron are not known, however. On theoretical grounds Wahl was able to build up a hypothesis to account for the change in acidity of hemoglobin on absorbing oxygen which is related to the equally important function of transporting carbon dioxide in the reverse direction. From a stereochemical standpoint the Wahl hypothesis is weak.

The closest approach to hemoglobin of a synthetic compound capable of carrying oxygen reversibly is the iron-indigo compound of Kunz and Kress (8), synthesized by the reaction of iron carbonyl with indigo in a pyridine solution. Carbon monoxide was evolved and a yellowish-red compound was obtained containing one atom of iron per molecule of indigo.



A pyridine solution of this compound absorbed oxygen, the color shifting at the same time from red to green. One molecule of oxygen was absorbed for each atom of iron, and on the application of a vacuum the oxygen was evolved and the color reverted to the original red. Carbon monoxide destroyed the ability of the solution to absorb oxygen. After several cycles this ability to absorb and release oxygen was lost owing to a gradual, irreversible oxidation of the organic material.

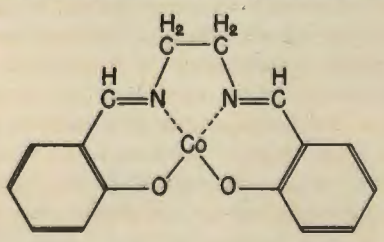
Hemoglobin has never been used for the manufacture of oxygen, although the patent of Sinding-Larsen (9) indicates that attempts have been made to do so. The body, of course, has mechanisms whereby the hemoglobin is constantly regenerated and it would appear that the rapid deterioration of the hemoglobin would make any in vitro process impracticable. The Kunz and Kress compound also cannot be used in this

manner owing to the rapid, irreversible oxidation of the material. Other inorganic compounds have been used, of course, for the recovery of oxygen from the atmosphere, barium oxide being the most noteworthy. The barium oxide process was investigated in some detail toward the close of the last century by the Brin brothers and others. It received a great deal of attention in the years around the turn of the century but was completely abandoned by 1920 in favor of the liquid air process. Considering the vastly improved engineering materials now available and the information regarding the conditioning of air now at hand, there is reason to believe that the Brin process might well operate economically in competition with the liquid air process. The principal disadvantage of the Brin process is the necessity of maintaining a considerably elevated temperature, of the order of 600°. The advantages of a chemical which would reversibly absorb and release oxygen at room temperature are obvious.

Bivalent cobalt salts in the presence of ammonia have the property of absorbing large quantities of oxygen. There is formed during this absorption a polynuclear coordination compound having a peroxo linkage between two cobalt atoms, Co-O-O-Co. On acidification the oxygen is expelled from this material and it is possible to use it for the recovery of oxygen from the atmosphere. A critical study of the process was made by Gluud, Keller, and Nordt (10) who concluded that such a process could never succeed commercially—principally because of irreversible oxidation of some cobalt to the trivalent state during each cycle. Warne and Woolcock (11) have applied the process to the manufacture of oxygen, using hexamminocobaltous perchlorate and a somewhat different technique; the oxygen was first caused to unite with the complex cobalt compound with the simultaneous expulsion of ammonia and then expelled from the peroxo cobalt compound so formed with the simultaneous reunion with ammonia. The equilibrium was shifted to and fro by controlling the concentration of ammonia, a mechanism for doing this being described. This process is indeed ingenious but there is no record of its actual utilization commercially.

Numerous binuclear cobalt ammoniates have been described by Werner and others (12), but a detailed examination covering many of these compounds has failed to reveal any in which the compounds are reported to reversibly absorb and release oxygen by a change in temperature or pressure.

The organic cobalt compound disalicylalethylenediimine cobalt first



mentioned by Pfeiffer, Breith, Lübbe, and Tsumaki (13) and later studied in more detail by Tsumaki (14), is quite unique in this respect. Tsumaki found that it absorbed about 3.5 per cent in weight of oxygen, corresponding more or less to a ratio of cobalt to an oxygen molecule of three to one. He found that the oxygen was expelled from the compound by heating it to 100° in a stream of carbon dioxide. Although it would appear from the work of Tsumaki that the compound once it had absorbed and given off its oxygen might be used again in the same manner, a careful reading of the Tsumaki papers discloses that he apparently did not appreciate the significance of such a cyclic behavior or prove that it was possible. It is strange that Tsumaki failed to grasp the significance of the material as he failed to mention either the possibility of using it for the recovery of oxygen from the atmosphere or of discovering means whereby the oxygen could be expelled and collected without the use of carbon dioxide.

The work reported in this series of papers is devoted almost entirely to this particular compound and to materials derived from it in various ways. The work was directed specifically to the utilization of the material for the commercial production of oxygen and many of the aspects of the problem of academic importance were deferred for future investigation. Broadly, the phases of the problem which were investigated and are being reported are: (1) the satisfactory manufacture of the material on a large scale, (2) the exact chemical constitution and the structure of the material, (3) the physical properties with particular reference to the ability to absorb and release oxygen, (4) the modification of the physical properties of the compound by the introduction of substituents, (5) the methods whereby it can be used for the generation of oxygen, and (6) the effective life and the economics of its utilization in the manufacture of oxygen.

Extensive studies were made of the methods of preparing the oxygen-carrying compound (designated Co-Ox) especially for its large-scale manufacture. The proper conditions were found for eliminating the side reactions, of securing consistently a material having the maximum oxygen-carrying capacity, of increasing the yield, and of decreasing the cost. There are several ways in which this cobalt compound can be made and the number of factors affecting the quality of the product is surprisingly large. These are dealt with in detail in Paper II.

Paper III deals with the chemical and physical properties of disalicylalethylenediimine cobalt. The exact composition of the material was determined as far as the difficulty of purifying the material permitted. It was discovered that water is present in the molecule in the ratio of one molecule of water to two cobalt atoms. Various proofs of the presence of this water are presented, including its direct determination by means of the Karl Fischer reagent and the synthesis under anhydrous conditions (Paper IV) of an inactive, orange material which becomes the oxygen carrier when treated with water. Apparently the water molecule acts as a bridging group to hold the two cobalt atoms, each surrounded by the quadridentate, chelating molecule of disalicylalethylenediimine, together

in a binuclear compound. The compound is thus actually bi-(disalicylalethylenediimine)-μ-aquo-dicobalt. There are two vacant positions in this compound, properly oriented to each other to permit the absorption of oxygen to form a peroxo group. The mechanism and stereochemistry of this are discussed in Paper III.

A red, inactive isomer of bi-(disalicylalethylenediimine)-μ-aquo-dicobalt was also found and the conditions for its formation and conversion to the active isomer are reported. Bi-(disalicylalethylenediimine)-μ-aquo-dicobalt was found to absorb nitric oxide and nitrogen dioxide also but not carbon monoxide and other gases. The compound is paramagnetic and becomes diamagnetic on absorbing oxygen. The rate of oxygenation of bi-(disalicylalethylenediimine)-μ-aquo-dicobalt at various temperatures and oxygen pressures was determined. The apparatus used for such measurements on this and the other compounds studied is described in Paper XIII and the data on the parent compound is presented in Paper III.

Numerous related compounds were prepared from substituted salicylaldehydes. Of the few which possessed the property of reversibly absorbing and releasing oxygen, those from 3-nitrosalicylaldehyde, 3-methoxysalicylaldehyde, 3-ethoxysalicylaldehyde and 3-*n*-butoxysalicylaldehyde absorbed oxygen more rapidly than the parent compound and were made the subject of more detailed studies, Papers V, VI, and VII. A few other compounds were prepared which were active toward oxygen, notably those derived from the various methylsalicylaldehydes, 3-chlorosalicylaldehyde, and *o*-hydroxyacetophenone, but the rate at which they absorbed oxygen was low. These compounds and the compounds which were found to be inactive are described in Paper VIII.

Substitution in or for the ethylenediamine portion of the molecule of disalicylalethylenediimine invariably produced a cobalt compound inactive toward oxygen. A variety of other diamines (Paper IX) and monamines (Paper X) were tried, all without success.

An interesting group of oxygen-carrying materials was obtained by starting with mixtures of aldehydes, for example salicylaldehyde and 3-methoxysalicylaldehyde. The physical properties of the cobalt derivatives depart considerably from those of the materials from the pure aldehydes owing to mixed crystal formation. This work is described in Paper XI. In Paper XII it is shown that an unsymmetrical Schiff's base of ethylenediamine cannot be made.

The concluding paper of the series deals with the engineering aspects of the manufacture of oxygen using these compounds. The compound may be used in a stationary bed or it may be used continuously by circulating the solid oxygen carrier from an oxygenation chamber to a deoxygenation chamber and back. Experience with each type of operation is reported in Paper XIV.

Several difficulties arose in the utilization of the material in the manufacture of oxygen from air. The material underwent a deep-seated, irreversible oxidation, deteriorating to about 50 per cent of its original, oxygen-carrying capacity in five thousand cycles. This is far too rapid

to be economic. The material is a light, fluffy powder which is an excellent heat insulator; the oxygenation-deoxygenation reaction is accompanied by a large heat of reaction, of the order of 20,000 calories per mole of oxygen. Consequently heat transfer during the cyclic operation becomes a major factor in the design of equipment. The material undergoes a change in density on oxygenation and the continued working of the crystal in cyclic operation, combined with its somewhat greasy, graphitic surface character, cause a stationary bed of the material to set up to a very hard solid which makes its replacement in the reaction vessel difficult. The light, fluffy powder is exceptionally fine, is difficult to filter and quite toxic. The opinion at present is that the process cannot be a serious competitor of the liquid air process. Nor do any of the substituted materials give promise of longer life or better thermal conductivity.

Academically the problem is of great interest. The mechanism of the oxygen absorption must be different from that of hemoglobin and oxygen, since in the latter one molecule of oxygen is absorbed per atom of iron while in the cobalt compound the ratio is one molecule of oxygen per two atoms of cobalt. This is, however, sufficient resemblance of the material to biologically important metal-containing materials to make its further study of value.

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STUDIES ON OXYGEN-CARRYING COBALT COMPOUNDS

II. THE METHODS OF PREPARING BI-(DISALICYLALETHYLENEDIIMINE)-μ-AQUO-DICOBALT, CO-OX

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The compound, bi-(disalicylalethylenediimine)-μ-aquo-dicobalt, was first prepared by bringing together simultaneously in an aqueous-alcohol reaction medium, cobalt acetate, ethylenediamine and salicylaldehyde (1). Somewhat later the same procedure was used and the product recrystallized from benzene and chloroform (2).

Our preliminary studies indicated that if care was used in the preparation, the oxygen-carrying capacity of the product could be greatly raised from the value of 3.5 per cent first reported. The formation of the cinnamon-colored, oxygen-carrying compound was complicated by the formation under certain conditions of three by-products, olive green, black, and red in color, all of which were found to be inactive toward oxygen.

The olive by-product was formed on prolonged contact of the cinnamon-colored, oxygen-carrying material with hot water, either during the formation of the material or during its drying. When heated at 200° in a vacuum it was partially reconverted to the active compound. It was also formed when the filtrate from a preparation of the cinnamon-colored, active compound was evaporated. The presence of any free acid, especially when hot, promoted the formation of the olive by-product.

The black by-product was formed from the cinnamon-colored, oxygen-carrying compound by contact with oxygen in the presence of alcohol. The black compound could not be deoxygenated. By washing the oxygen-carrying compound with dilute alcohol, or better with water, the formation of the black by-product was minimized.

The red by-product resulted from the action of alkali on the cinnamon-colored, oxygen-carrying compound. It was found to be isomeric with the oxygen-carrying compound but inactive toward oxygen. This compound is discussed in more detail in Paper III.

The theoretical value for the oxygen-carrying capacity of Co-Ox is 4.79 per cent, that is $\frac{32}{2(325)+18}$, 325 being the molecular weight of the cobalt-organic portion of the material and 18 being the molecular weight of the molecule of water associated with each two cobalt atoms (*see* Paper III for a discussion of this). Material of capacity greater than 4.7 per cent is therefore quite satisfactory. Somewhat higher capacities were found in certain instances; this was probably due to the adsorption

of oxygen on the finely powdered, oxygen-carrying material since the excess seldom exceeded 0.2 per cent and was rapidly lost at atmospheric pressure.

The earlier method of preparing this material was that of reacting simultaneously, solutions of cobalt acetate, ethylenediamine, and salicylaldehyde, in the molecular ratio of 1, 1, and 2, respectively, this reaction being most easily effected in water and alcohol so that the final solution contained about 60 per cent alcohol. It is possible in this procedure to form, in addition to Co-Ox, other compounds, principally cobalt salicylaldehyde, cobalt ethylenediamine salts, and the Schiff's base, disalicylalethylenediimine. Unless care was exercised the product was relatively impure. It was possible, however, by controlling a number of factors properly, to prepare a material having a high oxygen-carrying capacity by this method and the method was satisfactory for the preparation of the material in large quantities. Purer material was obtained by first preparing the Schiff's base, disalicylalethylenediimine, a beautifully crystalline, bright yellow solid. This Schiff's base was dissolved either in alkali or in alcohol and treated with an aqueous solution of a cobalt salt, yielding the same product as before but in a purer state.

Besides securing the stoichiometric ratio of the reactants, it was necessary in order to obtain a good product of high oxygen-carrying capacity to secure optimum conditions with respect to certain other factors, principally the rate at which the reactants were mixed, the volume of solvent used, the nature of the solvent, the temperature, the time of standing, the exposure to air while standing, the effectiveness of the washing after filtration, the speed at which the material was dried, and the temperature of drying. All of these factors were investigated and are discussed below.

For convenience the two methods just discussed are designated *Method A* (direct mixing of all three reactants) and *Method B* (preliminary preparation of Schiff's base).

Obviously it was desirable to eliminate the use of alcohol in the preparation. Numerous experiments with Method *A*, the direct mixing method, showed that when carried out without alcohol, the product was always of low oxygen-carrying capacity. Method *B* could, however, be carried out without alcohol and a satisfactory product obtained. The Schiff's base, disalicylalethylenediimine, was made by the addition of salicylaldehyde to a diluted, aqueous solution of ethylenediamine. To a solution of this yellow Schiff's base in a sodium hydroxide solution was then added a solution of a cobalt salt. It was found essential that the solution contain somewhat less than the two molecules of sodium hydroxide theoretically required since the alkali produced by hydrolysis of the salt of the Schiff's base or any excess sodium hydroxide added caused the formation of the red, inactive form of disalicylalethylenediimine cobalt.

It was desirable to use a cheaper salt of cobalt than the acetate, for example, the chloride, nitrate, or sulfate. When using a cobalt salt of a strong acid it was necessary to neutralize the strong acid liberated,

otherwise the cobalt compound was formed only to the extent of 20 to 30 per cent. The neutralization was made with sodium acetate or with sodium hydroxide or carbonate; with the latter two it was essential that a deficiency be used to prevent the formation of the inactive, red isomer.

In the interests of conserving space the numerous experiments which led to the conclusions stated above will not be described, but each of the various factors involved in the preparation are discussed in more detail and the working directions given for the best procedures developed.

A third method, designated *Method C*, was also of some interest. In it the pyridine addition compound, a red, crystalline solid, was first prepared and then rendered active toward oxygen by expelling the pyridine by heating the material in a vacuum. This procedure did not yield a product of high oxygen-carrying capacity and the method is impracticable for the preparation of large amounts of material.

It was observed that on the addition of a solution of a cobalt salt to a solution of the Schiff's base, disalicylalethylenediimine, there was first formed an orange precipitate which quickly changed over to the cinnamon-colored oxygen-carrier. This orange compound was isolated in pure form by carrying out the reaction under anhydrous conditions; it is discussed in detail in Paper IV.

FACTORS INVOLVED IN THE PREPARATION OF Co-Ox IN PURE FORM

Using Method *A* or Method *B* satisfactory material can only be prepared if certain factors are closely controlled.

FACTOR 1.

In some of our earlier work the Co-Ox produced was badly contaminated by the olive-colored by-product, even when the salicylaldehyde was distilled prior to use. It was found that the commercial salicylaldehyde contained some hydrochloric acid which distilled with the aldehyde. This acid was easily eliminated by treating the aldehyde with solid sodium bicarbonate or calcium carbonate prior to distillation. When distilled salicylaldehyde free from hydrochloric acid was employed, no trouble from this source was encountered.

The olive-colored material is produced by the prolonged action of water on Co-Ox particularly in the presence of free acid.

FACTOR 2.

In Method *A* it was possible to introduce three contaminating products by departing from strictly equivalent amounts of the reactants or by working in such a manner that the three reactants did not come together at the same time. Thus cobalt and salicylaldehyde formed a yellow, insoluble compound under about the same conditions in which Method *A* was carried out. Again, ethylenediamine and cobalt salts reacted to give compounds which contaminated the preparation. On the other hand, ethylenediamine and salicylaldehyde gave the yellow Schiff's

base which was only slightly soluble (about 4 per cent) in cold alcohol and less soluble in aqueous-alcohol mixtures. It was evident, therefore, that if the proper amounts of the reactants were not used, contamination would arise from three sources. A little consideration of this immediately showed that it was particularly important that the ethylenediamine and salicylaldehyde be added in exactly the ratio of one to two and that at least enough cobalt be present to react with the amine and aldehyde. A slight excess of cobalt acetate did no harm and was even desirable since the excess of cobalt acetate remained in solution and was subsequently washed out.

When the reaction was carried out observing these considerations, no further trouble from this source was encountered.

The neatest way found to insure the correct ratio of ethylenediamine to salicylaldehyde was to first prepare the yellow condensation product of the two, as is done in Method *B*. This, however, introduced another step.

Closely connected with the excess of the reagents is the matter of the order in which the reagents are added. Obviously, in Method *A*, since any two of the reagents may react to cause a contamination, it is necessary to get all of the reagents mixed as rapidly as possible. When following Method *A* it was found best to add the ethylenediamine and the salicylaldehyde, both previously diluted with about their own volume of 60–70 per cent alcohol, simultaneously to the cobalt acetate solution, stirring with all the vigor and effectiveness possible, and to maintain the stirring until the mass had set up to a thick paste, a matter of about 45 seconds.

In Method *B* it was most convenient to add the hot, aqueous solution of cobalt acetate to a hot, alcohol solution of the condensation product. The alcohol solution was cooled slightly as otherwise violent boiling occurred when the aqueous solution was added. The addition was carried out as rapidly as possible with effective stirring or shaking.

FACTOR 3.

The temperature of the solutions at the time of mixing in Method *A* had apparently little effect as preparations made with hot solutions were no better in yield or oxygen-carrying capacity than those made in the cold. In Method *B* the reaction was practically limited to hot solutions because of the relative insolubility of disalicylalethylenediimine in cold alcohol.

FACTOR 4.

When using water-alcohol mixtures as the reaction medium the oxygen-carrying capacity dropped when the alcohol concentration was made above 60 per cent. Under strictly anhydrous conditions other compounds are formed which yield Co-Ox on treatment with water (Paper IV). In addition, the formation of the black by-product on contact with air became more serious at the higher alcohol concentrations.

FACTOR 5.

If the volume of the aqueous-alcohol mixture used as solvent was too large the compound failed to form completely and the yield fell. In order to minimize the loss of product because of this, the volume of the solvent mixture was decreased as much as feasible. On the other hand, decreasing the volume too far caused the formation of contaminating products which decreased the oxygen-carrying capacity. In Method *A* the best condition found was that volume of 60 per cent alcohol which would just hold the required cobalt acetate in solution at room temperature. This required about three liters of the mixture per gram mole of cobalt acetate. The volume of alcohol used to dissolve the other two reagents then was small in comparison to the volume used to dissolve the cobalt acetate and did not sufficiently change the composition of the solvent to be of consequence.

In Method *B* it was found most convenient to dissolve the condensation product in 95 per cent alcohol. Attempts were made to dilute this solution of the condensation product with water to give a solvent mixture containing 60–70 per cent alcohol. This was done very carefully in order to prevent the precipitation of the yellow condensation product. By dissolving the cobalt acetate in that volume of water, which on addition to the solution of the condensation product in 95 per cent alcohol gave a mixture containing about 60–70 per cent alcohol, excellent results were obtained. On adding the aqueous solution of cobalt acetate (heated to a temperature of about 95°) to the alcohol solution of the condensation product cooled slightly below its boiling point, some cinnamon-colored material was formed immediately and the remainder precipitated within a minute or two. Since the product was obtained in high yield and of high capacity, the local concentration of the alcohol at the point of precipitation must have been about that necessary to give the cinnamon-colored material without any of the red by-product; apparently also the hot water raised the temperature of the solvent so that no yellow condensation product was thrown out to contaminate the cinnamon-colored material.

Attempts to isolate more of the oxygen-carrying compound from the filtrate after centrifuging off the cinnamon-colored material failed, owing to the formation of the olive-colored compound during the evaporation.

FACTOR 6.

Contact with air at the time of the reaction in methods *A* or *B* or during the period of standing before centrifuging lead to the formation of the black by-product, probably a trivalent cobalt compound. This material was soluble in water and alcohol and passed into the filtrate on filtering and washing. It was formed only when the material was still wet with the aqueous-alcohol mixture. The formation of this black material did not affect the yield appreciably, but its formation was minimized by sweeping the oxygen out of the reactants before mixing and by

maintaining an atmosphere of an inert gas over the reaction mixture during the period of standing before centrifuging. It was found equally satisfactory to cover the reaction mixture with a layer of aqueous-alcohol during the period of standing.

FACTOR 7.

The period of standing after the reaction and before centrifuging had little effect on the yield or oxygen-carrying capacity of the product. About fifteen minutes were required for the completion of the reaction; further standing did no harm. Thus, preparations filtered after fifteen minutes were identical with preparations which stood one hour, five hours, overnight, or one week.

FACTOR 8.

In Method *A* the reaction was usually carried out at room temperature and no advantage accrued from running the reaction at higher temperatures. The temperature rose about 15° during the reaction. The temperature fell during the period of standing so that cooling was not necessary. Since in Method *B* the reaction was carried out hot, the mixture was cooled before centrifuging. It was not necessary to cool this reaction before filtering; in fact, filtering hot was faster and aided in drying.

FACTOR 9.

As mentioned under Factor 6, a black by-product was formed when the cinnamon-colored material came in contact with air while still wet with alcohol. Washing with aqueous-alcohol mixtures further promoted the conversion of the material to this black by-product. It was found much better to wash immediately with water rather than with aqueous-alcohol mixtures. Water washed out the black material and left a uniform cinnamon-colored material, which was stable toward air.

FACTOR 10.

It was found that the washing of the precipitate could not be effectively carried out on the centrifuge. The cake was therefore removed from the centrifuge basket, broken up, stirred with wash water until a uniform slurry was produced, and the mixture again centrifuged. It was found best to repeat this a second time.

Continued washing with water did not finally give a clear filtrate. A reaction between the water and compound took place slowly giving the olive by-product which passed into the filtrate.

FACTOR 11.

One of the most important factors in the preparation of the oxygen-carrying, cinnamon-colored material is that of drying. Small preparations made in early, small scale, laboratory studies were dried in a steam heated, Fischer type, vacuum drying pistol. Larger amounts of material

were dried in a vacuum drying oven consisting of a horizontal brass tube, 12 inches in diameter and 20 inches long, enclosed in an electrically heated oven. When material was placed in this oven more than a half inch deep, the drying was very slow and the preparations were generally of low oxygen-carrying capacity. As mentioned under Factor 10 a reaction between water and the oxygen-carrying compound occurred. This reaction was slow at room temperature but was much more rapid when the wet compound was hot. During the slow drying process a considerable portion of the cinnamon-colored material was converted to the olive material and the oxygen-carrying capacity greatly decreased.

In another attempt to dry the centrifuged and washed compound, a stream of warm, dried carbon dioxide was passed over the compound. Although this worked fairly well it was still too slow to be satisfactory.

For handling one pound batches of compound, four glass tubes 1.25 inches in diameter and 36 inches long were finally used. These were set up vertically, surrounded by heating jackets and evacuated with an oil pump, properly protected with condenser, trap, and drying train. The material was broken up and placed in the tubes in small pieces. A total of one pound of material could be dried in this manner in three to four hours. The water was pulled off quite rapidly and the compound underwent very slight alteration during this time.

The drying process was materially assisted by pressing the water from the freshly filtered product. Using a hydraulic press and a pressure of about one ton per square inch, some 70 per cent of the moisture in the cake was expelled. Only a short time was necessary then for the vacuum drying and the oxygen-carrying capacity of the product was very high.

It was found possible to dry the cake at atmospheric pressure by exposing thin layers to 250 watt, reflector type drying bulbs at a distance of about six inches. This procedure worked best when most of the water was removed from the cake by pressure. The oxygen-carrying capacity never exceeded 4.5 per cent when the material was dried in this manner.

PREFERRED METHODS FOR THE PREPARATION OF Co-Ox

METHOD A. DIRECT MIXING OF REACTANTS

In a 1.5 gallon crock place 250 g. (1 mole plus 0.9 g. excess) of cobalt acetate, $Co(C_2H_3O_2)_2 \cdot 4H_2O$ and 3 l. of 60 per cent ethyl alcohol, sp. gr. 0.91. Agitate until all of the salt has dissolved. If a clear solution does not result it should be filtered. Prepare a mixture of 100 ml. of ethyl alcohol and 60.1 g. (1 mole) of ethylenediamine by weighing out that amount of aqueous ethylenediamine solution needed (ethylenediamine is marketed as an aqueous solution containing about 70 per cent ethylenediamine). Prepare a mixture of 244.2 g. of salicylaldehyde and approximately 250 ml. of alcohol (if any doubt exists about the purity of the salicylaldehyde it should first be treated with solid calcium carbonate, filtered, and then distilled). With a broad paddle agitate the cobalt acetate solution as vigorously as possible, and add the ethylenediamine solution. Add immediately and as rapidly as possible the salicylaldehyde

solution, maintaining the stirring at a vigorous rate. Continue to stir vigorously until the mixture sets up to a reddish-brown paste. Cover the mass with a half inch layer of water and allow to stand at least fifteen minutes.

Centrifuge off the solid and continue centrifuging until the mother liquor is completely removed. Add three portions of about 250 ml. of water, allowing the liquid to be centrifuged off each time before making the next addition. Remove the cake from the centrifuge basket and mix it up thoroughly with 1.5 l. of water so that no large particles remain and a uniform slurry is obtained. Filter again by centrifuging. Remove the cake and wash again in the same manner. Finally centrifuge as dry as possible. The filtrate will never run through clear but will be light brown in color. Break up the cake into small pieces, arrange in thin layers and dry at 100° in a good vacuum.

METHOD B. FROM THE SCHIFF'S BASE

Preparation of Disalicylalethylenediimine. Dissolve 244 g. (2 moles) of salicylaldehyde in 1 l. of boiling, 95 per cent ethyl alcohol. Stir and add 60.1 g. (1 mole) of ethylenediamine, measured by weighing out that amount of aqueous ethylenediamine solution needed. In 20–30 seconds the mass becomes solid with a bright yellow, crystalline material. Cool the reaction mixture, and filter on a Buchner funnel. The product may be spread out in thin layers on absorbent paper to dry. It may be recrystallized from 6 l. of hot 95 per cent alcohol or used without further purification. It will dissolve somewhat more rapidly in the next step if not allowed to dry out. Yield: about 255 g. or 95 per cent.

Preparation of Co-Ox, Using Alcohol. Dissolve 268 g. (1 mole) of disalicylalethylenediimine in 10 l. of boiling 95 per cent alcohol. In another vessel dissolve 250 g. (1 mole plus 0.9 g. excess) of cobalt acetate, $Co(C_2H_3O_2)_2 \cdot 4H_2O$, in 1.5 l. of boiling water; filter if a clear solution does not result. Turn off all burners. Cool the alcohol solution slightly below its boiling point, and with vigorous stirring, pour the hot cobalt acetate solution into the alcohol solution as rapidly as permissible. Vigorous boiling occurs and a considerable volume of alcohol vapors are evolved. Some compound forms immediately, and the reaction mixture sets up to a red-brown, pasty solid after about ten minutes. Cool the mixture below 30°. Continue with the filtration and remaining operations as described in the second paragraph of the procedure of Method *A*. Yield: 270 g., 80 per cent; oxygen-carrying capacity 4.7 to 4.8 per cent.

Preparation of Co-Ox, Without Using Alcohol. Dissolve 268 g. (1.0 mole) of finely ground disalicylalethylenediimine, 79.5 g. (2.0 moles less 0.5 g.) of sodium hydroxide, and 5 g. of sodium acetate, $NaC_2H_3O_2 \cdot 3H_2O$, in 3 l. of boiling water. The solution of the disalicylalethylenediimine requires from ten to twenty minutes and depends on the state of subdivision of the material and the agitation given the mixture. When the solution is complete, except possibly for the presence of a small amount of yellow scum on the surface of the solution, add to the solution 238 g. (1.0 mole

of cobalt chloride $CoCl_2 \cdot 6H_2O$) dissolved in 500 ml. of hot water. Agitate the solution vigorously during the addition of the cobalt salt solution. Continue with the filtration and remaining operations as described in the second paragraph of the procedure of Method *A*. Yield: 300 g., 90 per cent; oxygen-carrying capacity: 4.7 to 4.8 per cent.

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2. Tsumaki, *Bull. Chem. Soc. Japan*, 13:252. 1938.

STUDIES ON OXYGEN-CARRYING COBALT COMPOUNDS

III. THE COMPOSITION AND CHEMICAL PROPERTIES OF BI-(DISALICYLALETHYLENEDIIMINE)-μ-AQUO-DICOBALT, CO-OX

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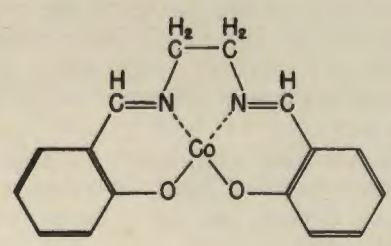
Obviously nothing in the simple composition of disalicylalethylenediimine cobalt predicates or explains the remarkable behavior of the



compound in reversibly absorbing and releasing oxygen. Since the oxygen and the cobalt combine in the ratio of one molecule of oxygen to two atoms of cobalt and because of the improbability of the oxygen molecule becoming dissociated in the process, it is likely that the attachment of the oxygen molecule to the cobalt is as a peroxo group

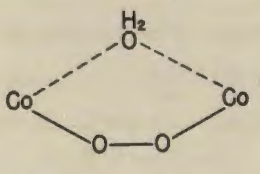
Co-O-O-Co

As indicated in Paper I of this series, compounds of this type are well known among the coordination compounds of cobalt, the peroxo group functioning as a bridging group to hold together the cobalt atoms of these so-called "polynuclear compounds." Usually in the polynuclear compounds containing peroxo bridging groups there is present a second or third bridging group, a hydroxyl, amino, or nitro group.

Disalicylalethylenediimine occupies four of the coordination positions of the cobalt atom. Cobalt, however, invariably has the coordination number six. It becomes pertinent, therefore, to inquire if all of the coordination positions are not filled in this compound, if perhaps there is not also present in the molecule a bridging group, water or possibly hy-

[1] The work by Diehl and Chao was done at Purdue University during the fall of 1938.

droxyl, tying the cobalt atoms together and arranging a convenient place for the oxygen molecule to enter to form a peroxo bridge:



That water is necessary for the formation of the oxygen-carrying compound is shown in Paper IV dealing with the reaction of disalicylalethylenediimine and cobalt chloride under anhydrous conditions. Under anhydrous conditions an orange material, inactive toward oxygen, is formed which yields the oxygen-carrying compound on treatment with water. The presence of the water in the compound could not be proved or disproved by chemical analysis owing to the lack of a satisfactory method of recrystallizing the compound for purification. Nor was a solvent found in which the molecular weight could be determined by the freezing or boiling point methods. Direct proof of the presence of the water was obtained, however, by heating the material in anhydrous pyridine, collecting the distillate and determining by means of the Karl Fischer reagent the water expelled from the compound and distilled over with the pyridine. Each of these aspects of the problem are discussed in some detail in the following sections.

ANALYSIS AND COMPOSITION

Unfortunately Co-Ox is insoluble in water and most organic solvents and cannot be recrystallized for purification. It is soluble in pyridine and chloroform but crystallizes from these solvents with solvent of crystallization in which form it does not absorb oxygen. On removal of the solvent of crystallization by heating the material in a vacuum only part of the activity toward oxygen is restored. Thus, the compound must be prepared sufficiently pure for analysis when first formed. Using the best methods of preparation found (Paper II of this series) and using nickel-free cobalt salts, several highly pure specimens of Co-Ox were made. The oxygen-carrying capacities of these materials ranged from 4.70 to 4.80 per cent, agreeing well with the theoretical value of 4.79 for a compound possessing a half molecule of water per cobalt atom.

These materials were analyzed for cobalt, nitrogen, carbon, and hydrogen as carefully as possible. The results on a single preparation were in excellent agreement, but minor variations were found from preparation to preparation indicating that an absolutely pure compound was not obtained. On one preparation the molecular weight as calculated from the analysis for cobalt was 326, from the analysis for nitrogen 335, carbon 332, and hydrogen (assuming 14 hydrogen atoms present)

328. On another sample the analysis for cobalt indicated 332 and for nitrogen 328. On a third the analysis for nitrogen gave 332. The theoretical molecular weight of the anhydrous molecule, $C_{16}H_{14}O_2N_2Co$, is 325, that of a hemihydrate 334, and that of molecule containing a hydroxyl group as a bridging group (μ-hydroxyl) 333.5. The results indicate that some other material is present but the uncertainty in the analytical results from preparation to preparation is just sufficient to prohibit drawing a categorical conclusion. The analysis of one sample for hydrogen gave: 4.32, 4.27, 4.33, 4.36, 4.28, aver.: 4.30. This indicated that no hydrogen was present beyond that required by the simple, anhydrous formulation, 4.31 per cent, significantly below a μ-hydroxyl material which would contain 4.35 per cent hydrogen or a hemihydrate 4.49 per cent hydrogen.

MOLECULAR WEIGHT

It is obvious that the question of the binuclear composition of the compound could be settled positively if the molecular weight of the material could be determined. Disalicylalethylenediimine cobalt is somewhat soluble in chloroform and in pyridine. It crystallizes from these solvents with chloroform or pyridine of crystallization so that in solution the material undoubtedly ties up some solvent. In a cryoscopic determination of molecular weight in these solvents this would be negligible with respect to the total volume of solvent or could be corrected for on the basis of one molecule of solvent for each cobalt. However, in both chloroform and in pyridine by the boiling point method, a depression of the boiling point was obtained instead of the expected elevation. This can only be explained on the basis of water being expelled from the compound and increasing the vapor pressure.

An apparatus was constructed for measuring the lowering of the freezing point of pyridine. It consisted of two cells, one for pure pyridine and the second for the pyridine solution, a ten junction thermopile, mechanical stirrers in each cell and in the surrounding toluene bath which was contained in a large Dewar flask. The bath was cooled by dry ice. Suitable radiation shields were inserted. A White potentiometer was used to measure the potential of the thermopile. This apparatus worked very well with naphthalene and the Schiff's base, disalicylalethylenediimine. When the cobalt compound was dissolved in the pyridine, however, reproducible and steady values could not be obtained for the freezing point. It appeared again that water was being drawn from the compound even in the cold, assisted undoubtedly by the preferential coordination of pyridine to the cobalt forming a compound containing two molecules of pyridine per cobalt atom.

The solubility of Co-Ox in a variety of solvents was determined roughly. The solubility in the three most promising solvents, benzene, ethylenedibromide, and bromoform, was too low to give a significant change in the freezing point or the boiling point.

DIRECT DETERMINATION OF WATER PRESENT IN Co-Ox

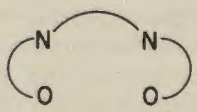
The most direct attack on the question of the presence of water in the oxygen-carrying compound appeared to be the expulsion of the water at a relatively high temperature and its gravimetric determination in a suitable desiccant. Experiments along this line indicated that such water if present was not expelled at temperatures up to 190°. Slow decomposition occurred above 170° with the liberation of salicylaldehyde. The formation of salicylaldehyde from the Schiff's base requires water but this can be considered only qualitative evidence.

The failure of the efforts to determine the molecular weight of the material cryoscopically provided the key to the solution of the problem. Since pyridine apparently expels the water from the compound it was only necessary to heat the material with pyridine and determine the water distilled with the pyridine. The water was determined by the Karl Fischer reagent. The results on one sample were 2.21 and 2.26 per cent water, on another sample 2.47 per cent; the calculated amount of water for a half molecule of water per cobalt atom is 2.69. The details of this work are described in the experimental section.

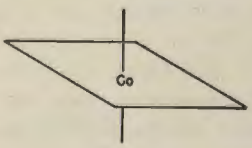
Polynuclear cobalt compounds of the type proposed here are well known (7) but the bridging groups previously reported have been the hydroxyl (-OH), the amino ($-NH_2$), the nitro ($-NO_2$), the peroxo (-O-O-), and the oxo (-O-). This appears to be the first case of a water molecule acting as the bridging group.

STEREOCHEMISTRY

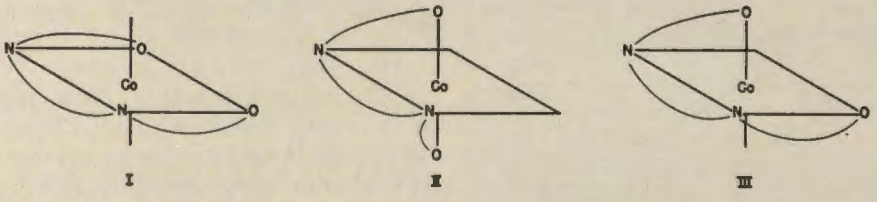
Having proved the presence of a half molecule of water per cobalt atom, the binuclear character of the compound is inescapable and it becomes of interest to inquire into the stereochemical arrangement of the various molecules about the cobalt atoms. There exist three ways in which the organic molecule, disalicylalethylenediimine, can be arranged about the cobalt atom. Adopting for the disalicylalethylenediimine molecule the shorter symbol



and utilizing the usual abbreviated octahedron for designating the six coordination positions about the cobalt atom

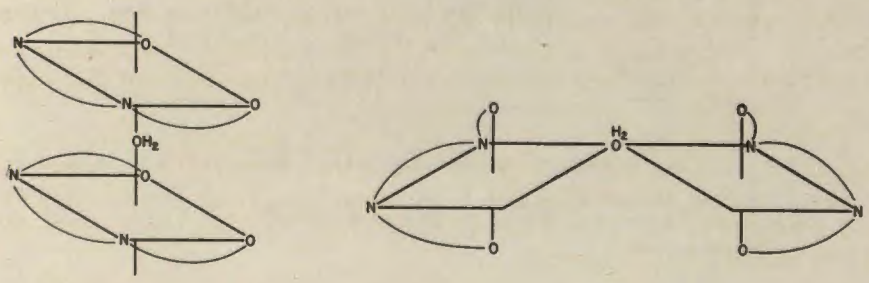


these arrangements are

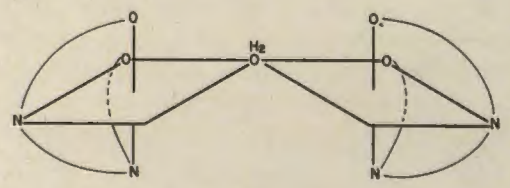


I II III

All of these forms are more or less strain free as shown by models, but form I is perhaps the most stable. Considering the half molecule of water present per cobalt atom to function as a bridging group between two cobalt atoms, the structure of the various forms then becomes

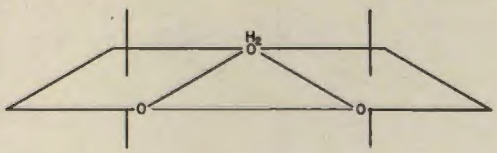


Ia IIa

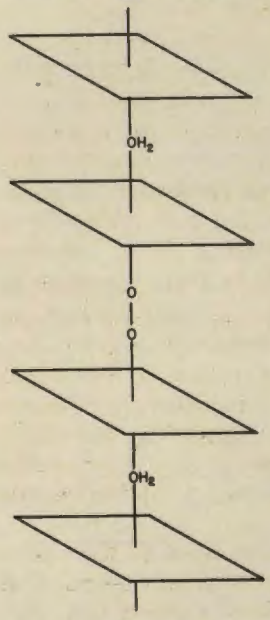


IIIa

In each of the three structures, Ia, IIa, and IIIa, the sixth coordination position of each of the cobalt atoms is left vacant. In structures IIa and IIIa these positions are adjoining and there is just sufficient room for an oxygen molecule to slip into the empty space to form a peroxo bridge.

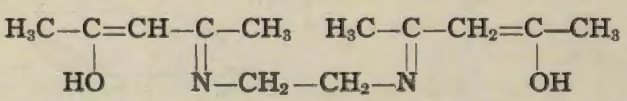


In structure Ia there is no such convenient space provided for the oxygen molecule and oxygen absorption could only then occur intermolecularly which requires that the crystal structure be so arranged that this may occur as in IV. This structure is much less probable for the oxygen-carrying compound than structures IIa and IIIa, in which the favorable orientations are forced by the *cis*-arrangement of the bridging water molecule and the vacant coordination positions made accessible to the oxygen molecule.

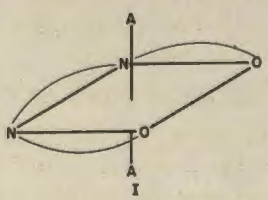


IV

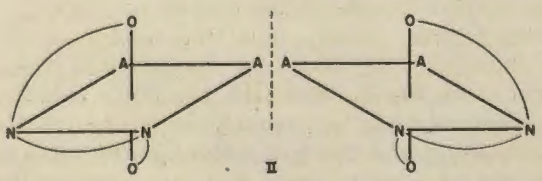
Similar isomeric compounds were prepared by Morgan and Smith (4) who prepared three isomeric forms of diacetylacetoethylenediimine cobalt, two of which they were able to resolve, owing to their unsymmetric nature. Representing the chelating compound



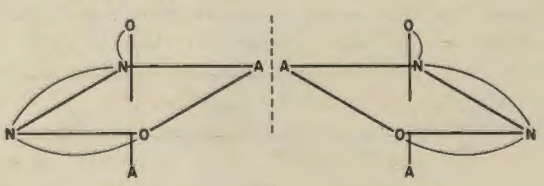
appropriately, these isomers corresponded to



I



II



III

A = NH_3, Cl^-, etc.

identical with the possible forms suggested for Co-Ox with the difference that in Co-Ox the aromatic rings lend less flexibility to the molecule.

On this basis there should exist three isomeric forms of bi- (disalicylalethylenediimine) -μ-aquo-dicobalt, two of which should carry oxygen and one of which should be inactive. As a result of X-ray diffraction and absorption spectra studies, it was shown that the material prepared in an aqueous medium from the Schiff's base is identical with the material prepared in an aqueous-alcohol medium by direct mixing of the reactants. The bright red, inactive compound discussed below is unquestionably one of the isomeric forms. A third form has not yet been found.

THE BRIGHT RED, INACTIVE ISOMER

As mentioned in Paper II of this series, a bright red, crystalline compound may be obtained in place of the cinnamon-colored, oxygen-carrying compound when an excess of alkali is present during the synthesis. It appeared desirable to obtain a pure preparation of this bright red material and to ascertain its character. A satisfactory procedure was devised for converting Co-Ox into the inactive isomer by heating it with alcoholic potassium hydroxide. Analyses indicated that the material was isomeric with Co-Ox and probably also contained a half molecule of water.

It was observed that the red compound was converted to the active form by grinding it with mineral oil and a crystalline material such as lucite or potassium chloride; if the mineral oil was washed out with benzene the material reverted to the inactive form.

X-ray diffraction (powder) patterns showed that Co-Ox and its red, inactive isomer are distinctly different compounds. No detailed crystallographic study of the material was attempted.

THE EFFECT OF GASES OTHER THAN OXYGEN ON Co-Ox

Co-Ox did not change in color or gain in weight when placed in carbon monoxide at atmospheric pressure. This is rather surprising in view of the extreme avidity of hemoglobin for carbon monoxide and of the Kunz and Kress iron-indigo compound for carbon monoxide.

Co-Ox absorbed nitric oxide rapidly with the evolution of heat. The compound turned dark blue in color and experienced a gain in weight corresponding to one molecule of nitric oxide per cobalt atom. The nitric oxide addition product was very stable; only about one-third of the nitric oxide was expelled at 170° in a vacuum. The bright red, inactive isomer of Co-Ox also absorbed nitric oxide and although the rate of absorption was much smaller, the total gain corresponded to about 1.35 molecules of nitric oxide per cobalt atom. The absorption of nitric oxide in this case was almost completely reversible.

In the case of nitrogen dioxide, the Schiff's base as well as Co-Ox and the inactive, red isomer absorbed nitrogen dioxide. All three materials absorbed approximately four molecules of gas per molecule of compound. Apparently the reaction in this case is different from the absorp-

tion of oxygen and nitric oxide, the nitrogen dioxide attacking the organic material, possibly at the double bond.

Nitrous oxide was not absorbed by Co-Ox or by its red isomer.

Deoxygenated Co-Ox is paramagnetic. It is quite possible therefore that the first step in the oxygenation of Co-Ox is a magnetic coupling of the cobalt compound with the oxygen molecule. It would be expected then that other paramagnetic gases should be absorbed by disalicylalethylenediimine cobalt but that diamagnetic gases should not be absorbed. The failure of the diamagnetic gases, carbon monoxide, carbon dioxide, nitrous oxide, nitrogen, and argon to be absorbed is in agreement with this. On the other hand, nitric oxide and nitrogen dioxide, which are paramagnetic, are absorbed, although the evidence for the latter is confused by the action of the nitrogen dioxide on the organic portion of the molecule.

Carbon monoxide can be rendered paramagnetic by radiation with ultra-violet light of very short wave length and chlorine can also be rendered paramagnetic by exposure to certain radiation; it would be of interest at some time in the future to study the absorption of these gases under such conditions.

Assuming that the process by which the oxygen is absorbed by bi-(disalicylalethylenediimine)-μ-aquo-dicobalt is the two step process just outlined, it would appear that the second step has a large temperature coefficient since the rate at which oxygen is absorbed decreases as the temperature decreases. The magnetic interaction, however, should become stronger at lower temperatures and it is predicted that oxygen will be absorbed by the compound at very low temperatures, the magnetic interaction of the oxygen and the cobalt compound being sufficient to hold the oxygen and cobalt compound together without the further step involving the formation of a peroxo bridge.

MAGNETIC SUSCEPTIBILITY AND ELECTRONIC STRUCTURE

The magnetic susceptibility of oxygenated and deoxygenated Co-Ox was measured. The deoxygenated form was found to be paramagnetic and the oxygenated form diamagnetic. The molar susceptibility using the molecular weight of 334 which is that of one cobalt atom surrounded by one molecule of disalicylalethylenediimine of the deoxygenated form was 316×10^{-5} corresponding to an effective moment 2.73 Bohr magnetons. This indicates one free electron per cobalt atom.

One unpaired electron per cobalt atom is what would be expected in this material. The atomic number of cobalt is 27, the cobalt is bivalent, and to the 25 electrons around the cobalt atom are added, eight electrons from the four covalent linkages with the organic material and two from the oxygen of the bridging water molecule, making a total of 35, the odd $4p$ electron remaining unpaired. The oxygen molecule is paramagnetic, having two unpaired electrons. The coupling, then, of the cobalt compound with the oxygen molecule is probably by the pairing of the un-

coupled electrons, two cobalt atoms being required to pair off the two unpaired electrons of the oxygen molecule.

RATE OF OXYGENATION

The rate of oxygenation of bi-(disalicylalethylenediimine)-μ-aquo-dicobalt was determined using the gas volumetric method described in Paper XIV, *Method D.* The material was carried on circular fins attached to a vertical metal tube through which cooling water passed; the heat transfer was therefore excellent. The purity of the oxygen used was determined by the usual gas analysis method using the alkaline hydrosulfite reagent. Both cylinder oxygen and oxygen generated from potassium chlorate and manganese dioxide in a carefully evacuated generator were used. The retaining liquids in the apparatus were freed of air by boiling or by sweeping with oxygen and then protected from the air.

Using a constant temperature of 25–27°, the rate of oxygenation was determined at various pressures from 200 to 870 mm. of mercury. As will be seen from Figure 1, the rate of oxygenation decreased rapidly with pressure, and the per cent of oxygen taken up at infinite time, taken as four hours, also decreased. The point of inflection of the curves about corresponded to half saturation, and the time of half saturation values were therefore taken as a typical point for the comparison of the rates at different pressures. A plot of these times against pressure is given in Figure 2.

The rate of oxygenation was measured at various temperatures from −7° to +41°, holding the pressure constant at 510 mm. of mercury. As will be seen from the results which are presented graphically

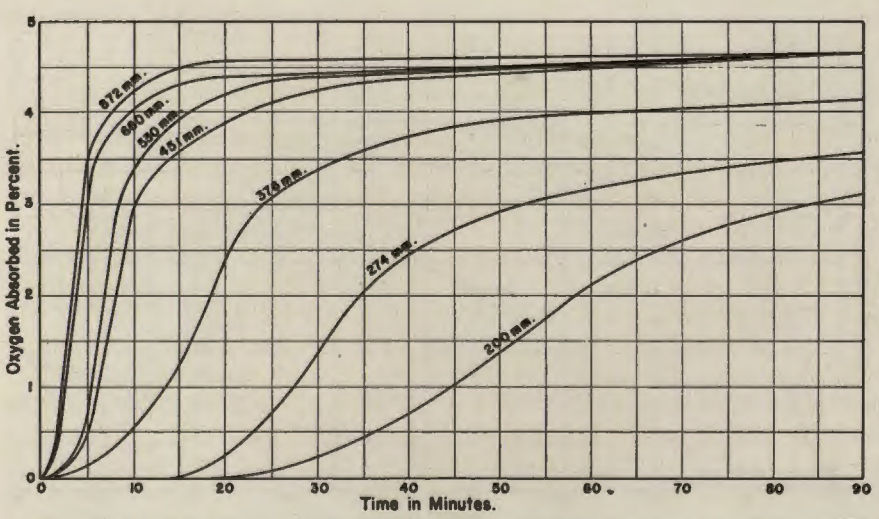


FIG. 1. Rate of oxygenation of bi-(disalicylalethylenediimine)-μ-aquo-dicobalt in oxygen at various pressures. Temperature constant: 25–27°. Pressure in mm. of Hg.

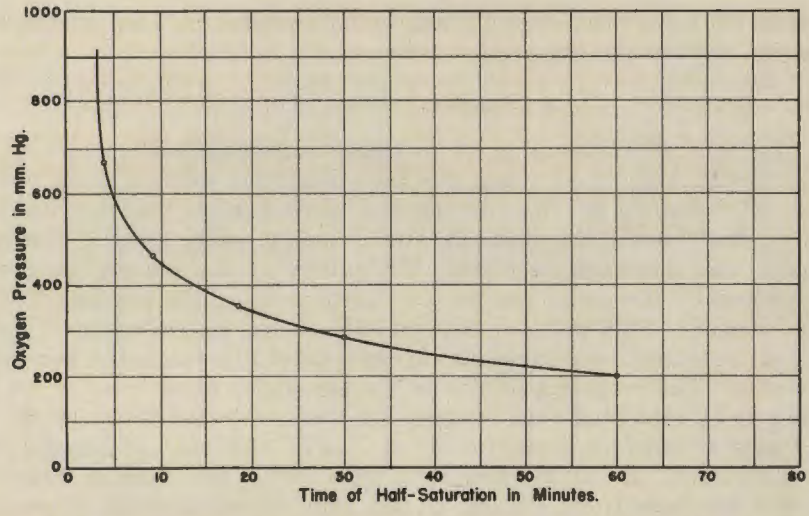


FIG. 2. Effect of pressure on the rate of oxygenation of bi-(disalicylalethylenediimine)-μ-aquo-dicobalt.

in Figures 3 and 4, the rate of oxygenation fell off at higher temperatures and again at lower temperatures. The experimental error in this series of measurements was rather high and the data could not always be exactly reproduced, owing probably to some factor related to the activation of the material. It became apparent, however, that the optimum temperature of oxygenation was close to 20° and that increasing the pressure of oxygen beyond 900 mm. of mercury could not significantly

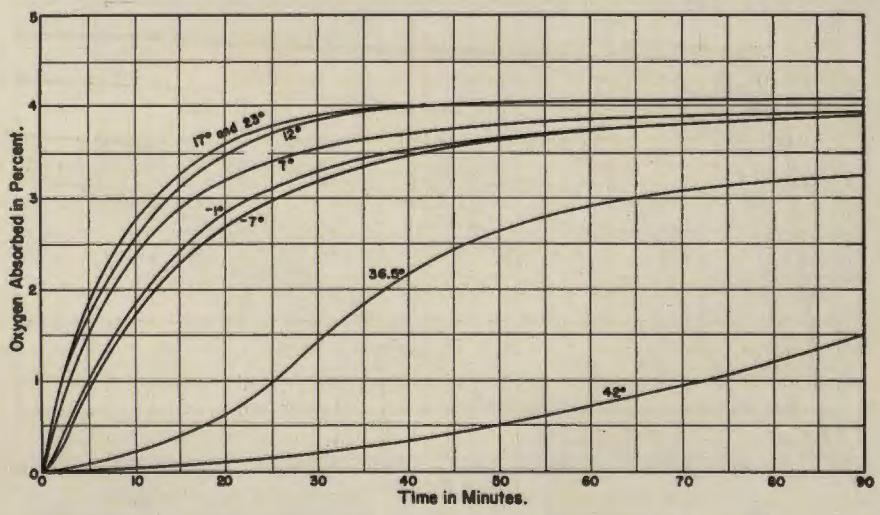


FIG. 3. Rate of oxygenation of bi-(disalicylalethylenediimine)-μ-aquo-dicobalt in oxygen at various temperatures. Pressure constant: 510 mm. Hg.

decrease the time which was required to oxygenate the compound. Judging from the curves a temperature of 45° to 50° should be ample to effect the deoxygenation of the compound at atmospheric pressure. This was found experimentally to be true.

Assuming that the oxygen pressure-oxygenation rate relationship will hold when air is used as the oxygenating agent if the air is circulated freely, an air pressure of 100 pounds should oxygenate the compound in eight to ten minutes.

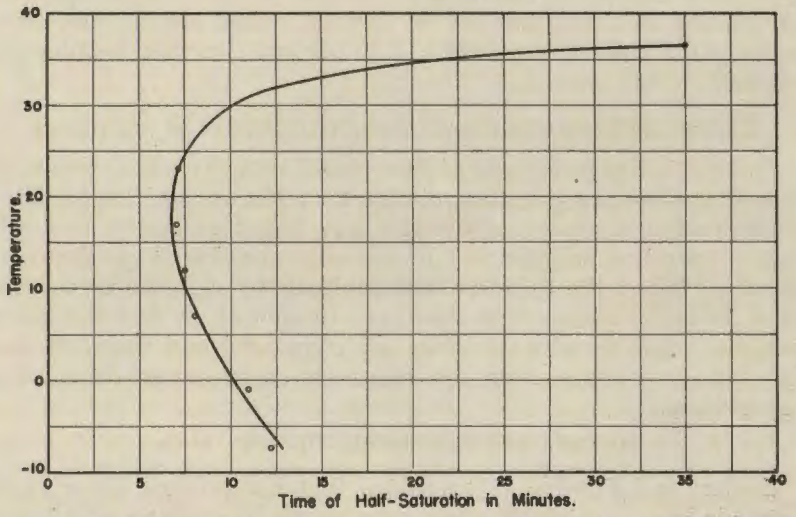


FIG. 4. Effect of temperature on the rate of oxygenation of bi-(disalicylalethylenediimine)-μ-aquo-dicobalt.

The time required for oxygenation was found to be much greater if the heat of oxygenation was retained in the material. The temperature of the material rose about 15° under adiabatic conditions.

The rate of oxygenation of bi-(disalicylalethylenediimine)-μ-aquo-dicobalt in oxygen was also determined at various temperatures with a second apparatus; see Paper XIV, Figure 1.

THE OLIVE BY-PRODUCT

As indicated in Paper II, Co-Ox is converted by water, particularly when hot and in the presence of acid, to an olive-colored material which does not absorb oxygen. In the absence of other obvious reasons, the preparation of a batch of material having an oxygen-carrying capacity below normal is probably due to the formation of some olive by-product.

It was found possible to convert a preparation of Co-Ox which had been only somewhat impaired by the formation of the olive by-product back to the oxygen-carrying compound by heating it in a vacuum at 170°. Thus, for example, a preparation of Co-Ox having an oxygen-carrying capacity of only 4.42 per cent was raised in capacity to 4.75 per cent

by heating for 45 minutes in a vacuum at 195°. This is not only important with respect to the initial preparation of the oxygen-carrying material but also to the regeneration of oxygen-carrying material which has become impaired in use as a result of having absorbed water. However, studies made on oxygen-carrying material, which had been put through 4,400 oxygenation-deoxygenation cycles and had deteriorated in oxygen-carrying capacity to about 20 per cent of its original value, was found to be capable of some regeneration by such a heat treatment, although the improvement found was hardly enough to be of commercial significance. Thus, heating in a vacuum for 60 minutes raised the oxygen-carrying capacity of the material from 0.9 to 1.46 per cent; further heating caused a decrease in the capacity.

CYCLIC OXYGENATION AND DEOXYGENATION IN SOLUTION

The iron-indigo compound of Kunz and Kress (5) was shown to carry oxygen reversibly in a pyridine solution for a few cycles. Bi-(disalicylalethylenediimine)-μ-aquo-dicobalt also was found to absorb and release oxygen in pyridine solution but it was only possible to go through the cycle once. When the pyridine was replaced by chloroform no enrichment of the air by oxygen was observed. In view of the fact that pyridine causes the expulsion of water from the compound and therefore breaks up the binuclear compounds, this failure to carry oxygen in solution is not surprising.

MISCELLANEOUS PROPERTIES OF Co-Ox

Thielert and Pfeiffer (6) reported that many ferric inner complex compounds have a catalytic effect causing the luminescence of 3-aminophthalhydrazide in an alkaline solution containing hydrogen peroxide. Bi-(disalicylalethylenediimine)-μ-aquo-dicobalt has this same effect, a minute amount of the compound causing a brilliant bluish luminescence.

Bi-(disalicylalethylenediimine)-μ-aquo-dicobalt, in either the oxygenated condition or the deoxygenated condition, acquires an electrostatic charge with great ease. In merely pouring a small quantity of the material from a piece of paper, it becomes so highly charged that it clings to the paper with considerable tenacity. Adjacent particles of the Co-Ox repel each other. The ease with which the charge is acquired and its size appear to be rather unusual.

Co-Ox has been described variously in the preceding pages as being of a maroon or cinnamon color. More precisely it matches the R3/4 shade of the Munsell Book of Color.

EXPERIMENTAL WORK

METHOD OF ANALYSIS

The usual Kjeldahl method failed to yield consistent or correct values for nitrogen on either disalicylalethylenediimine or its cobalt derivative. Satisfactory results were secured by treating the sample first

with dilute sulfuric acid (1:1) and then evaporating to concentrate the sulfuric acid. The digestion with a little selenium oxychloride catalyst and the usual distillation were then carried out. Presumably direct digestion with concentrated sulfuric acid converted the nitrogen to oxides of nitrogen which escaped. Preliminary treatment with dilute acid first hydrolyzed the Schiff's base and the ethylenediamine formed then yielded ammonium sulfate on digestion.

Disalicylalethylenediimine was carefully purified by recrystallization and analyzed by this modified Kjeldahl procedure. Found: 10.35, 10.34, 10.47, 10.48, per cent nitrogen; calculated for $C_{16}H_{16}O_2N_2$, mol. wt. 268: 10.44 per cent nitrogen.

The obvious method of determining cobalt in these compounds, that of evaporating with sulfuric acid and weighing the cobalt sulfate produced, is unsatisfactory owing to a very serious creeping of the solution over the walls of the crucible. All of the volumetric methods in the literature for cobalt were found to be unsatisfactory except the potentiometric titration in ammoniacal citrate solution with ferricyanide (1,2). The ferricyanide method yielded excellent results on known cobalt solutions. Primary standard cobalt sulfate was prepared for reference by evaporating chloropentamminocobalti chloride with sulfuric acid and heating the resulting cobalt sulfate at 550° for several hours. The organic cobalt compounds were decomposed by boiling with a mixture of dilute nitric and sulfuric acids, and after complete decomposition, the cobalt solution was treated with a large amount of citric acid, made ammonical, and titrated potentiometrically with ferricyanide.

EFFECT OF THE PRESENCE OF NICKEL

Since any conclusions drawn from the results of analyses of Co-Ox are highly subject to error if impurities are present it became pertinent to inquire into the fate of any nickel present in the cobalt salts used in the preparation of Co-Ox. The nickel in various cobalt preparations was determined by the method of Feigl and Kapulitzas (3) in which the cobalt is converted to a cobalticyanide complex, the excess hydrogen peroxide and cyanide and the nickel cyanide complex being destroyed by the addition of formaldehyde, and the nickel precipitated with dimethylglyoxime. Found: 0.12, 0.21 per cent nickel in "C. P." cobalt chloride. Found: 0.16, 0.22 per cent nickel in Co-Ox prepared from this cobalt chloride.

Apparently nickel is carried down about quantitatively during the formation of Co-Ox. That nickel salts alone yield a compound with disalicylalethylenediimine was quickly confirmed, the product being brown in color and very insoluble. Since nickel would not be measured with the cobalt in the ferricyanide method for cobalt it was apparent that a nickel-free compound would have to be prepared if the results for cobalt were to be of significance.

NICKEL-FREE COBALT CHLORIDE THROUGH SODIUM AMMONIUM COBALTINITRITE

A saturated solution of 1,200 g. of cobalt chloride was prepared and filtered. Enough acetic acid was added to make the solution about 25 per cent acetic acid. Approximately 1,000 g. of sodium nitrite was added as rapidly as possible. During the next two hours more sodium nitrite and acetic acid were added whenever the evolution of gas ceased. The solution was then allowed to stand about four hours, until the reaction was nearly completed. About 400 g. of ammonium chloride was then added. After standing overnight the yellow precipitate which had formed was filtered off and dried. This sodium ammonium cobaltinitrite was ignited to the oxide in a graphite crucible. The residue of oxide was leached with water several times and then dissolved in a little less than the theoretical amount of hydrochloric acid. The excess oxide was then filtered off, the solution evaporated down, and the cobalt chloride crystallized out. It was found that if the sodium salts were not leached out before dissolving the oxide, a double chloride of cobalt and sodium formed which was not satisfactory for our purpose. The cobalt chloride obtained was analyzed for nickel by the method of Feigl and Kapulitzas (3); no nickel was present.

RESULTS OF ANALYSES

Careful analyses were made of several different preparations of Co-Ox for nitrogen, cobalt, carbon, and hydrogen. The results on these analyses were good and indicated that the molecular weight of the compound is higher than that of the simple compound disalicylalethylenediimine cobalt, that is 325. The values for hydrogen are disconcerting in that they fail completely to show the half molecule of water per cobalt atom known to be present. As will be seen the values for hydrogen in the Schiff's base, disalicylalethylenediimine were quite satisfactory.

RED, INACTIVE FORM OF DISALICYLALETHYLENEDIIMINE COBALT

About 40 g. of Co-Ox, oxygen-carrying capacity 4.74 per cent, was treated with 200 ml. of 3 per cent potassium hydroxide in absolute alcohol. The mixture was boiled gently for 20 minutes. In the course of a few minutes the cinnamon-colored, oxygen-carrying compound was changed to a cherry-red, crystalline material. After cooling, the material was filtered and washed well with water, until the filtrate was colorless. The material was then pressed between layers of cloth in a hydraulic press exerting about 0.75 ton per square inch, which removed most of the water. It was then dried in a vacuum for 30 minutes at 100°. Yield: approximately quantitative; oxygen-carrying capacity: 0.02 per cent. Found: 8.30, 8.32, 8.42 per cent N by the modified Kjeldahl method; 17.75, 17.89, 17.67 per cent Co, by ferricyanide titration; calculated for $C_{16}H_{14}O_2N_2Co$, mol. wt.: 325: 8.61 per cent N, 18.13 per cent Co.

The cobalt-nitrogen ratio calculated from these data is almost exactly one to two but the results of both are low, the molecular weight calcu-

TABLE 1

SUMMARY OF RESULTS OF ANALYSES OF CO-OX AND OF THE SCHIFF'S BASE, DISALICYLALETHYLENEDIIMINE

Preparation	For	Percentage Found	Average Percentage	Calculated Molecular Weight
Co-Ox (V-18).....	N	8.43, 8.40, 8.32	8.39	334
	Co	18.02	18.02	326
	C*	57.9, 57.9, 58.1	57.97	332
	H	4.32, 4.27, 4.33, 4.30, 4.28	4.30	328
Co-Ox (V-23-A)...	N	8.56, 8.58, 8.59, 8.49	8.55	328
	Co	17.79, 17.76, 17.70	17.75	332
Co-Ox (V-23-B)...	N	8.15, 8.22, 8.24, 8.15	8.19	3.42
Schiff's Base.....	H	6.00, 6.00, 6.00, 6.00, 6.01, 6.12	6.02	6.00†
	C§	69.7, 70.6, 67.0, 72.0, 71.8, 71.75	70.46	71.5

* Oxides of nitrogen removed by sulfuric acid-chromate mixture.
† Theoretical value.
§ Oxides of nitrogen removed with reduced copper spiral.

lated from the nitrogen and cobalt analyses are 336.0 and 333.5, respectively, indicating some impurity, possibly a half molecule of water. It is apparent that this material is isomeric with the oxygen-carrying compound, Co-Ox.

During efforts to prepare a thin layer of the bright red compound on glass in order to measure its absorption spectrum, the bright red compound was ground with mineral oil and powdered lucite in an agate mortar. Strangely, its color changed from bright red to a brown of the same shade as the oxygen-carrying compound. After this transformation, the material absorbed oxygen turning black and released oxygen returning to the cinnamon brown color. It was also found that grinding the bright red compound with mineral oil and potassium chloride produced the same effect. No quantitative data were secured as to the quantity of oxygen taken up owing to the contamination of the bright red compound with mineral oil and potassium chloride or lucite. One preparation was washed with benzene to remove the mineral oil; after this treatment the material was again bright red in color and inactive.

THE DIRECT DETERMINATION OF WATER IN CO-OX

The apparatus shown in Figure 5 was used. The 500 ml., two-necked flask served as a distilling flask. The sample was introduced through the neck closed by the ground glass plug which was removed only very briefly for the introduction of the sample or of pyridine. The distilling head, thermometer, condenser, adapter, and titration vessel were

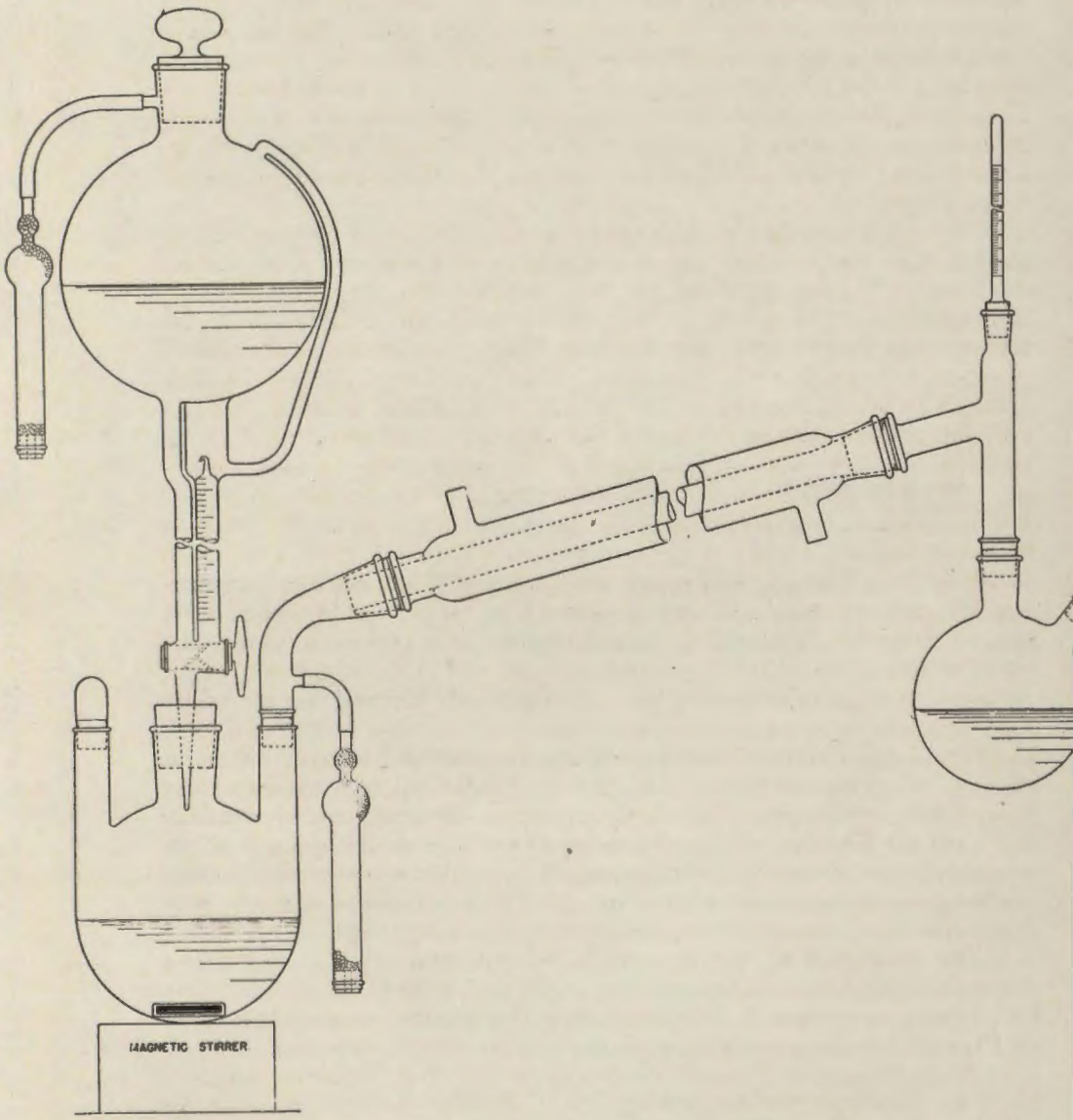


FIG. 5. Apparatus for the direct determination of water.

all connected by ground glass joints. The drying tube attached to the adapter was filled with anhydrous calcium sulfate. The titration vessel consisted of a 1 l. three-necked flask. The Karl Fischer reagent was dispensed from a Machlett buret, the tip of which passed through a rubber stopper inserted in the central neck of the flask. The third neck was closed by a ground glass stopper. The flask was emptied by suction through a tube inserted through this neck so that it was unnecessary to disassemble the apparatus at any time. The times the flasks were open to the atmosphere were held to the very minimum necessary to pipet in the samples. The solution in the titration vessel was stirred magnetically.

The determination was carried out by placing about 100 ml. of methanol in the titration vessel and about 100 ml. of pyridine in the distilling flask. The pyridine was then distilled into the titration vessel until about 30 ml. remained in the distilling flask. The methanol-pyridine solution was then titrated with the Karl Fischer reagent. Exactly 100 ml. of anhydrous pyridine was then added to the distillation flask and distilled until about 30 ml. remained. The solution was titrated with the reagent and this volume designated as the pyridine blank. A second 100 ml. of pyridine was then distilled as a check on this blank. The weighed sample and 100 ml. of pyridine were then introduced into the distilling flask and 100 ml. of pyridine distilled over, taking about 30 minutes. The solution was then titrated, representing the water in 100 ml. of pyridine plus the water derived from the compound, the latter being obtained by subtracting the pyridine blank. To make certain the water was expelled from the compound a further 100 ml. of pyridine was added, distilled, and titrated. The small volume required to titrate this portion in excess of the pyridine blank was added to the volume of reagent used for the water derived from the compound.

The Karl Fischer reagent was standardized by titrating 50.0 ml. portions of a standard water in methanol solution made by weighing water from a weight buret. A blank on the methanol was run on methanol put through identical treatment without added water. The anhydrous methanol was prepared by distillation from magnesium turnings treated with a little mercuric chloride. The anhydrous pyridine was prepared by distillation from freshly broken walnut potassium hydroxide.

The samples of Co-Ox were dried in a vacuum at 120° prior to the analysis.

Found on sample *A* (prepared from the Schiff's base by Method *B* of Paper II; oxygen-carrying capacity: 4.64 per cent): 2.21, 2.26 per cent water, on sample *B* (sample *A* recrystallized from benzene; oxygen-carrying capacity: 4.79 per cent): 2.47 per cent water; calculated for $C_{16}H_{14}O_2N_2 \cdot \frac{1}{2} H_2O$: 2.69 per cent water.

ACTION OF CARBON MONOXIDE ON CO-OX

Carbon monoxide was generated by the action of sulfuric acid on formic acid. The carbon monoxide was scrubbed with alkali and col-

lected over water, precautions being taken to eliminate all traces of air from the retaining liquid and apparatus. A weighed sample of Co-Ox was placed in a glass tube and the tube evacuated with an oil pump. Carbon monoxide was then admitted to the tube until atmospheric pressure was attained. After 18 hours at room temperature the color of the compound was unchanged and the material had not gained in weight.

ACTION OF NITRIC OXIDE ON CO-OX

Nitric oxide was generated by the action of sulfuric acid on sodium nitrite, washed with sodium hydroxide, and collected over water. A sample of deoxygenated Co-Ox was placed in a glass tube at room temperature, the tube evacuated with an oil pump, and nitric oxide then admitted to the tube until the pressure reached atmospheric pressure. The compound immediately turned a dark blue in color and absorbed nitric oxide with the evolution of considerable heat. The reaction was complete within three minutes. The sample gained 9.31 per cent in weight. On heating the material for 30 minutes in a vacuum at 100°, a loss in weight of 0.18 per cent was observed; on further heating at 174° for six hours in a vacuum a further loss in weight of 3.70 per cent occurred.

One molecule of nitric oxide per molecule of Co-Ox corresponds to a gain in weight of 8.98 per cent (mol. wt. Co-Ox: 334). The observed value 9.31 per cent is thus slightly higher than the figure for one mole of nitric oxide per one cobalt atom: this might be explained by the presence of some nitrogen dioxide in the nitric oxide used in the experiment (see below).

ACTION OF NITRIC OXIDE ON RED, INACTIVE DISALICYLALETHYLENEDIIMINE COBALT

A weighed sample of the bright red, inactive compound was placed in a tube, the tube evacuated with an oil pump, and nitric oxide admitted until atmospheric pressure was attained. The bright red color changed slowly to a dark brown, the reaction between this compound and nitric oxide being a great deal slower than the reaction between Co-Ox and nitric oxide. After about ten hours the material was found to have gained 12.5 per cent by weight. The dark brown, nitric oxide containing compound was then heated at 170° in a vacuum for six hours; a loss in weight of 12.22 per cent was observed. It appears therefore, that the nitric oxide is reversibly absorbed by the bright red compound.

ACTION OF NITROGEN DIOXIDE ON CO-OX AND INACTIVE DISALICYLALETHYLENEDIIMINE COBALT

Nitrogen dioxide was prepared by oxidizing dry nitric oxide prepared by the action of sulfuric acid on potassium nitrite. Sulfuric acid was slowly dropped on a 25 per cent solution of potassium nitrite. The nitric oxide evolved was dried over phosphorous pentoxide, and then

mixed with pure, dry oxygen in an Erlenmeyer flask containing glass beads cooled in an ice-bath. The gas condensed to a green-blue liquid, a mixture of nitric oxide and nitrogen dioxide. Oxygen was bubbled through the liquid until the color changed to a light tan. The liquid was stored at —20°. Weighed samples of disalicylalethylenediimine, of deoxygenated Co-Ox, and of inactive disalicylalethylenediimine cobalt were placed in a bottle and the bottle evacuated. Nitrogen dioxide was admitted slowly until atmospheric pressure was attained. After fifteen hours the samples were removed and weighed. All three materials had absorbed nitrogen dioxide and turned dark brown in color. Co-Ox picked up 56.1 per cent in weight, its red inactive isomer 48.7 per cent, and the Schiff's base 70 per cent. These gains correspond respectively to 3.97, 3.45, and 4.06 molecules of nitrogen dioxide per molecule of compound. It was apparent that the nitrogen dioxide attacked the organic molecule, undoubtedly at the double bonds and that the absorption was of a character entirely different from the absorption of oxygen or nitric oxide.

ACTION OF NITROUS OXIDE ON CO-OX

Nitrous oxide was prepared by heating a mixture of ammonium nitrate and sea sand to 200°. The gas was collected over water. Weighed samples of deoxygenated Co-Ox and its inactive, red isomer were placed in a glass tube, the tube evacuated, and nitrous oxide admitted until atmospheric pressure was attained. After ten hours the Co-Ox had gained 0.34 per cent in weight and the inactive isomer 0.18 per cent. The gains in weight absorbed were probably due to oxygen in the nitrous oxide derived from the water retaining liquid or possibly to nitrous oxide remaining mixed with the powdered materials on the boat. In any case nitrous oxide did not appear to be absorbed.

MAGNETIC SUSCEPTIBILITY MEASUREMENT

The magnetic susceptibility measurements were made with a Gouy type apparatus, using a field of about 12.000 gauss. A standard nickel chloride solution, 1.336 *M*, was prepared from Mond nickel and used for calibration. The volume susceptibility used was 5.332×10^{-6} per ml. (8). The sample tube was identical with the one described by Freed (8) being made from pyrex tubing 3 mm. in diameter with a thin uniform partition at the center. The tube was checked for magnetic symmetry at various field strengths. The usual precautions were taken to eliminate temperature effects and ferromagnetic impurities. The tube was calibrated before and after a measurement on the oxygen-carrying compound to insure that the field strength and other factors remained constant. A current of 22 amperes was used, well into the saturation region of the magnet.

The oxygen-carrying compound was prepared from cobalt sulfate prepared by decomposing chloropentamminocobalti chloride; in this way the absence of impurities of iron and nickel was guaranteed.

The following values were obtained for the molar susceptibilities:

Co-Ox, deoxygenated	$X = 316 \times 10^{-5}$
	$\mu = 2.73$
Co-Ox, oxygenated	$X = 18.3 \times 10^{-5}$
	$\mu = 0.656$
Red, inactive isomer	$X = 269 \times 10^{-5}$
	$\mu = 2.52$

The molecular weight of 334 was used in making these calculations, that is, the molecular weight of one cobalt atom, with its associated Schiff's base and half molecule of water.

DENSITY OF DEOXYGENATED AND OXYGENATED CO-OX

The densities of the deoxygenated and oxygenated forms of bi-(disalicylalethylenediimine)-μ-aquo-dicobalt were determined under toluene in a Geissler specific gravity bottle. The density was calculated from the density of toluene at the temperature of the determination and from the weight of toluene displaced by the compound.

Deoxygenated form: 1.526, 1.521
Oxygenated form: 1.618, 1.605

CYCLIC OXYGENATION AND DEOXYGENATION IN SOLUTION

The apparatus employed is pictured in Figure 6. Provision was made for saturating the solution containing the Co-Ox with air, and for then

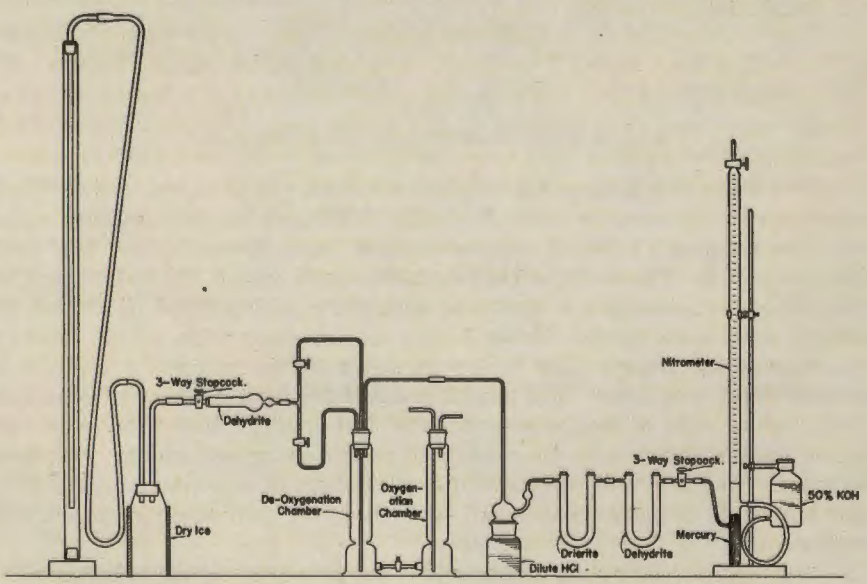


FIG. 6. Apparatus for testing cyclic operation of bi-(disalicylalethylenediimine)-μ-aquo-dicobalt in solution.

transferring the solution to another vessel in which the air was swept out of the solution with a stream of carbon dioxide gas. The gaseous mixture was then passed through a potassium hydroxide solution in a nitrometer where the carbon dioxide was absorbed and the remaining oxygen collected. By carrying out the oxygenation and deoxygenation steps in separate vessels, the danger of admitting air during the deoxygenation step was avoided. Provision was made for absorbing the solvent swept from the deoxygenation vessel by the carbon dioxide stream. The nitrometer used for measuring the evolved gases was filled with a 50 per cent solution of potassium hydroxide.

The procedure adopted in carrying out the oxygenation-deoxygenation cycle on a sample was as follows: A quantity of 240 ml. of pyridine, b.p. 113.5–114.5°/740 mm., was introduced into the oxygenation chamber. The apparatus was swept out with carbon dioxide until only microbubbles were observed to reach the top of the nitrometer. The sample of pyridine was then transferred into the deoxygenation chamber and the carbon dioxide bubbled through the pyridine until microbubbles were again obtained. This process required about 45 minutes. It was now assumed that the pyridine was freed of all previously absorbed gases.

A blank determination on the pure pyridine was made by transferring the solution to the oxygenation chamber and drawing air through the solution by means of a water aspirator for a definite period of time, usually five or ten minutes. The solution was again returned to the deoxygenation system and carbon dioxide bubbled through until all absorbed air was removed as shown by the size of the bubbles reaching the top of the nitrometer.

The pyridine was transferred again to the oxygenation chamber to which had been added a weighed sample of deoxygenated Co-Ox. Air was drawn through the pyridine solution by means of a water aspirator for the same period of time as in the blank run. The pyridine solution was transferred again to the deoxygenation chamber and carbon dioxide bubbled through the pyridine solution until all absorbed air was removed as shown by the microbubbles reaching the top of the nitrometer.

In some of the preliminary work oxygen gas was used in place of air. The gas obtained from the pyridine solution was rich in oxygen as demonstrated by the fact that a glowing splint was immediately ignited when placed in an atmosphere of the gas.

It seemed probable that some pyridine vapor was not absorbed by the sulfamic acid and anhydrone used in the purification train, and, therefore, was present in the gas finally collected in the nitrometer. This conclusion was drawn from the fact that in two instances when testing for oxygen with a glowing splint an explosion resulted inside the nitrometer. For the subsequent work the purification train described in the drawing was replaced by a gas wash bottle containing 1 *N* hydrochloric acid and followed by a U-tube containing anhydrous calcium sulfate and another containing anhydrous magnesium perchlorate. The gas collected was analyzed for oxygen. Results are shown in Table 2.

TABLE 2
SUMMARY OF RESULTS USING PYRIDINE
Volume of pyridine: 240 ml.

Experiment Number	Time of Saturation, Minutes	Gas Used	Weight of Sample, Grams	Volume Collected, Ml.	Oxygen Content, Percentage
1-A	10	O_2	blank	17.5	none, ignited glowing splint
1-B	10	O_2	0.8369	18.2	" "
2-A	10	O_2	blank	28.2	81
2-B	10	O_2	0.9660	28.1	86
3-A	10	air	blank	15.5	21
3-B	10	air	0.949	22.5	45
3-C	10	air	0.949	14.8	21
4-A	15	air	blank	14.2	21
4-B	15	air	0.979	22.5	57
4-C	5	air	0.979	14.2	20

The blank obtained was apparently the result of the absorption of air by pyridine since the oxygen content of the gas collected was about the same as that of air. Upon the introduction of the oxygen-carrying compound an additional amount of gas was obtained, which upon analysis was shown to contain more oxygen. Evidently the cycle cannot be repeated as the gas collected upon repeating the oxygenation-deoxygenation cycle had the same oxygen content as the blank.

The procedure was repeated using chloroform as the solvent. The drying agents following the deoxygenation chamber were replaced with a dry ice trap. These results are summarized in Table 3.

TABLE 3
SUMMARY OF RESULTS USING CHLOROFORM
Volume of chloroform: 240 ml.

Experiment Number	Time of Saturation, Minutes	Gas Used	Weight of Sample, Grams	Volume Collected, Ml.	Oxygen Content, Percentage
1-A	10	air	blank	27	29
1-B	10	air	0.5810	27	28.5
2-A	10	air	blank	28	26.8
2-B	10	air	0.4630	32	25

The results indicate that no increase in the oxygen content of the gas absorbed resulted when the compound was dissolved in chloroform. Evidently the chloroform itself has a preferential solubility for oxygen since even the blanks showed a higher percentage of oxygen than in air.

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[Further discussions of the "Studies on Oxygen-Carrying Cobalt Compounds" will follow in subsequent issues of the *Journal of Science.*]

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SUBJECT INDEX